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Award Number: DAMD17-01-1-0054

TITLE: Functional Analysis of p53 Acetylation in Prostate Tumor Suppression

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REPORT DATE: July 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2004	Final (1 Jul 01-30 Jun 04)

4. TITLE AND SUBTITLE
Functional Analysis of p53 Acetylation in Prostate Tumor Suppression

5. FUNDING NUMBERS
DAMD17-01-1-0054

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The tumor suppressor p53 becomes acetylated upon its activation. We show that MDM2 can promote p53 deacetylation by recruiting a complex containing HDAC1. The HDAC1 complex binds MDM2 in a p53-independent manner and deacetylates p53 at all known acetylated lysines *in vivo*. Fibroblasts over-expressing a dominant negative HDAC1 mutant display enhanced DNA damage-induced p53 acetylation, increased levels of p53, and a more pronounced induction of p21 and MDM2. These results indicate that acetylation promotes p53 stability and function. As the acetylated p53 lysine residues overlap with those that are ubiquitinated, our results suggest that one major function of p53 acetylation is to promote p53 stability by preventing MDM2-dependent ubiquitination, while recruitment of HDAC1 by MDM2 promotes p53 degradation by removing these acetyl groups. We also found that acetylation regulates p53 subcellular localization by promoting p53 nuclear export. Thus, acetylation controls p53 function at several levels. Finally, we report that MDM2 is also subject to acetylation. We present evidence that acetylation-deficient MDM2 mutant is defective in promoting p53 deacetylation and degradation. Our studies reveal protein acetylation as an important mechanism in regulating the p53-MDM2 tumor suppressor network.

14. SUBJECT TERMS
P53, acetylation, ubiquitination, MDMD2, HDAC

15. NUMBER OF PAGES
59

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Title: Functional analysis of p53 acetylation in prostate tumor suppression

Introduction:

The long-term goal of this research proposal is to understand the regulation and the functional importance of specific p53 acetylation in tumor suppression. The activation of p53 is a critical step in preventing oncogenic transformation and multiple types of tumor formation. The importance of p53 in prostate cancer has been illustrated by its critical role in the prostate gland apoptosis induced by androgen-ablation, a powerful therapy used to treat early stage, androgen-dependent prostate cancer. Further supporting the dominant role of p53 in suppressing prostate tumor progression, mutations in p53 are prevalent in majority of tumors that have progressed to a more advanced and hormone-refractory stage. Understanding the role of p53 acetylation could provide critical information on how activation of p53 is achieved. The data we have obtained during the grant period provide evidence that acetylation regulates p53 at several levels. We also demonstrated that MDM2, the key negative regulator for p53, is also subject to regulation by acetylation. Togethere, these results establish reversible acetylation as an important regulatory modification that controls the function of the p53-MDM2 tumor suppressor network.

Body:

I. Functional characterization of p53 acetylation and its regulation in response to cellular stresses

Briefly, we have obtained evidence that acetylation is a common modification associated with p53 activation. We showed that p53 invariably becomes acetylated upon its activation in response to a wide variety of activating agents. Furthermore, we found that p53 acetylation is transient and it is tightly regulated by the opposing forces of the acetyltransferase p300/CBP and deacetylases. We have identified a novel role of MDM2 in the negative regulation of p53 acetylation. We also uncovered the mechanism by which MDM2 achieves its inhibitory activity toward p53 acetylation. These results will lead to a better understanding of the regulation of p53 acetylation.

In the pursuit of the function for p53 acetylation, we generated p53 acetylation deficient mutants and began to investigate their activities. Although these mutants appear to have relatively normal transcriptional and apoptotic activities, they are resistant to MDM2 mediated -degradation. We have now obtained several lines of evidence that one key function of acetylation is to promote p53 stability.

The detailed description of the research progress can be found in the Appended two manuscripts (1 and 2).

II. Control of p53 subcellular localization by acetylation at C-terminus lysine residues

Briefly, we have obtained evidence that acetylation of p53 also influences p53 subcellular localization. By promoting p53 acetylation either by over-expressing acetyltransferase CBP or by inhibiting deacetylases, we found an accumulation of p53 in the cytoplasm. This cytoplasmic localization requires the lysine residues known to be subject to acetylation. Further, we showed that acetylation appears to work by neutralizing charge at the C-terminus of p53 thereby preventing p53 tetramerization and exposing p53 nuclear export. Our finding strongly support that acetylation controls p53 at several levels.

The detailed description of this part of study can be found in the Appended manuscript to be submitted for publication (3).

III. The regulation of MDM2 by acetylation

In the last part of our study, we have made significant progress in charactering the function of MDM2 acetylation.

1. MDM2 is subject to protein acetylation in vivo

By Mass Spectrometry, we have also successfully identified lysine residues in MDM2 that are subject to acetylation. By generating anti-acetylated MDM2 specific antibodies and site directed mutagenesis, we confirmed that MDM2 is acetylated at specific lysines in vivo (Figure 1).

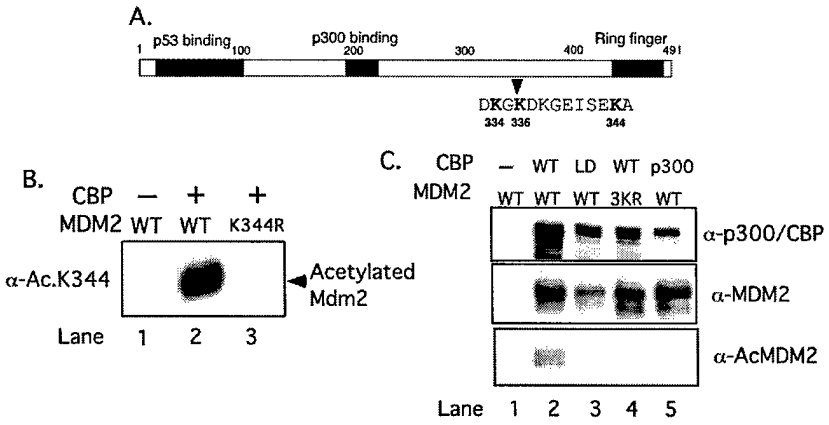

Figure 1. MDM2 is modified by acetylation. (A) A schematic diagram of MDM2. The three acetylated lysine residues identified by Mass Spectrometry are marked. **(B). The generation of acetylated MDM2 specific antibodies.** Peptides (14 amino acids) containing at either lysine 334, 336 or 344 (Ac-K334, Ac-K336 or Ac-K344) were synthesized and used to generate antibodies in rabbits. The acetylated MDM2 specific antibodies were obtained by sequential affinity purification using the unacetylated peptide column to remove immuno-reactivity to unmodified MDM2, followed by an acetylated peptide column. Anti-Ac-K344 was used here as an example. Unmodified recombinant MDM2 (without HAT), wild type or mutant MDM2, whose K344 was converted to arginine, were subject to *in vitro* acetylation by CBP (plus HAT) followed by immuno-blotting with the anti-Ac-K344. Note that this antibody only recognized wild type MDM2 that was acetylated by CBP (compare Lane 1 and 2) and it failed to react with the MDM2 mutant of which lysine 344 was converted to arginine (K344R, Lane 3). Thus, this antibody specifically recognizes MDM2 acetylated at lysine 344. Similarly, antibodies against acetylated MDM2 at lysine 334 (α-Ac-K334) or 336 (α-Ac-K336) were found to specifically recognize the acetylated MDM2 (data not shown). (C) **MDM2 is acetylated *in vivo*.** Expression plasmids for wild type or mutant MDM2 with lysine 334, 336 and 344 converted to arginine (3KR) were co-expressed with wild type CBP, acetyltransferase inactive CBP mutant (CBP-LD) or wild type p300. The acetylation status of MDM2 was then determined by immunoprecipitating with an antibody against MDM2 followed by immuno-blotting with a mixture of anti-acetylated lysine MDM2 antibodies (α-AcMDM2: a mixture of α-Ac-K334, α-Ac-K336 and α-Ac-K344). Note that these acetylated MDM2 specific antibodies recognized MDM2 when wild type CBP (Lane 2) but not its enzyme-deficient LD mutant (Lane 3) or p300 (Lane 5) was co-expressed. Importantly, these antibodies did not react with 3KR mutant in the presence of ectopically expressed CBP (Lane 4), further demonstrating the specificity of the antibody toward acetylated MDM2 at specific lysines. Similar results on MDM2 acetylation *in vivo* were obtained using antibodies against individual acetylated lysine residues (data not shown). The expression of p300, CBP and MDM2 were confirmed by immuno-blotting with their respective antibodies.

2. Endogenous MDM2 acetylation can be induced by UV-irradiation

To begin to investigate whether MDM2 acetylation is regulated. We determined the MDM2 acetylation level in response to DNA damaging agent treatment. As shown in Figure2, an apparent induction of MDM2 acetylation can be observed after UV-irradiation.

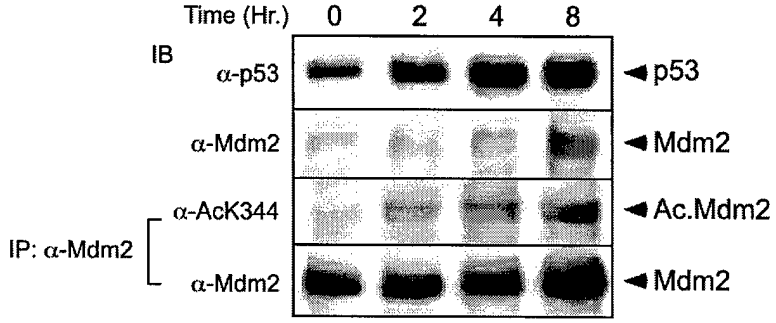

Figure 2. Endogenous Mdm2 acetylation is induced by DNA damage agent. A549 cell were irradiated by UV for 45 seconds (energy output equals to 75J/m2) and treated with TSA (5 uM) and nicotinamide (5mM) after UV irradiation. Cells were harvested at 0, 2, 4 and 8 hour time points. Cell extracts were analyzed by straight western blotting with anti-Mdm2 and anti-p53 to detect Mdm2 protein level and p53 protein level and anti-Mdm2 immunoprecipitation western blotting with anti-acetylated K344.Mdm2 antibody to detect the acetylated Mdm2 level.

3. Acetylation deficient MDM2 mutant is defective in inducing p53 deacetylation and degradation.

To begin to investigate the functional significance of MDM2 acetylation, MDM2 mutants that are deficient in acetylation were generated by coverting the acetylation acceptor lysine residues to arginine. Functional analysis shows that these mutants are defective in promoting p53 deacetylation and degradation.

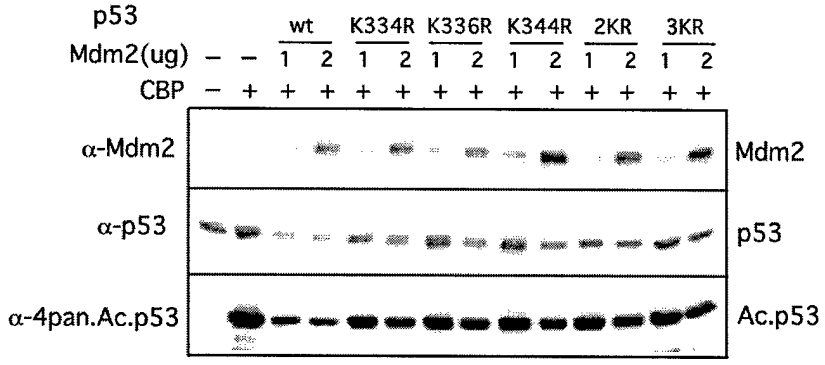

Figure. 3. **The effect on p53 stability and deacetylation by wild type and KR (lysine->arginine) mutants Mdm2.** MEF p53 -/- cells were transiently transfected with expression plasmids of CBP, p53 and 2 different doses of either wild type, single KR, double KR or triple KR mutants Mdm2 as indicated. 24 hours after transfection, cells were harvested and cell extracts were analyzed by straight western blotting with anti-Mdm2, anti-p53, and anti-4 pan acetylated p53 antibodies to detect Mdm2 protein level, total p53 protein level and acetylated p53 level, respectively.

Key Research Accomplishment

1. We have identified acetylation as an important modification associated with p53 activation in response to a wide variety of agents.
2. We have obtained evidence that p300 and CBP can function as the p53 acetyltransferases in vivo.
3. We have uncovered a role of MDM2 in negatively regulating p53 acetylation by at least two different mechanisms. MDM2 can bind CBP and inhibits its acetyltransferase activity and it can also recruit HDAC1 to promote p53 deacetylation. The inhibitory activity of MDM2 toward p53 acetylation can be reversed by tumor suppressor $p14^{ARF}$.
4. We have identified the key acetylation sites in p53 and showed that these lysines are also the targets of MDM2 mediated ubiquitination.
5. We have obtained evidence that acetylation promotes p53 stability, possibly, by competing with the MDM2-mediated ubiquitination.

6. We have identified the key acetylation sites in p53 and showed that these lysines are also the targets of MDM2 mediated ubiquitination. We have obtained evidence that acetylation promotes p53 stability, possibly, by competing with the MDM2-mediated ubiquitination
7. We have found that full acetylation of p53 can lead to its nuclear exit.
8. We have identified lysine residues in MDM2 that are subject to acetylation.
9. We showed that acetylation is likely required for MDM2 to promote p53 deacetylation and degradation.

Reportable Outcome:

Reportable Outcome:

1. Ito, A., Lai, C.-H*, Zhao, X.*, Huacani, M.R., Saito, S., Appella, E., and **Yao, T.-P#.** p300/CBP mediated- p53 acetylation is commonly induced by p53 activating agents and inhibited by mdm2. **EMBO J.** 20, 1331-1340 (2001)

2. Ito, A. Kawaguchi, Y., Lai, C.-H, Kovacs, J. J., Higashimoto, Y., Appella, E., and **Yao, T.-P#.** An MDM2-HDAC1 complex controls p53 acetylation and stability by the coupling of p53 acetylation and ubiquitination (Submitted to EMBO. J., under revision)

3. Kawaguchi, Y., Ito, A., Appella, E., and **Yao. T.-P#.** Control of p53 subcellular localization by charge modification at C-terminus lysine residues (in preparation)

4. Lai, C.-H, Basrus, V., Ito, A. Appella, E., Yao T.-P#. Regulation of MDM2 activity by CBP-mediated acetylation. (In Preparation)

Conclusion:

Our study has provided evidence that protein acetylation is an important regulatory modification that controls the function of p53-MDM2 tumor suppressor network.

The EMBO Journal Vol. 20 No. 6 pp. 1331–1340, 2001

p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2

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The tumor suppressor p53 is activated in response to many types of cellular and environmental insults via mechanisms involving post-translational modification. Here we demonstrate that, unlike phosphorylation, p53 invariably undergoes acetylation in cells exposed to a variety of stress-inducing agents including hypoxia, anti-metabolites, nuclear export inhibitor and actinomycin D treatment. *In vivo*, p53 acetylation is mediated by the p300 and CBP acetyltransferases. Overexpression of either p300 or CBP, but not an acetyltransferase-deficient mutant, efficiently induces specific p53 acetylation. In contrast, MDM2, a negative regulator of p53, actively suppresses p300/CBP-mediated p53 acetylation *in vivo* and *in vitro*. This inhibitory activity of MDM2 on p53 acetylation is in turn abrogated by tumor suppressor p19^{ARF}, indicating that regulation of acetylation is a central target of the p53–MDM2–p19^{ARF} feedback loop. Functionally, inhibition of deacetylation promotes p53 stability, suggesting that acetylation plays a positive role in the accumulation of p53 protein in stress response. Our results provide evidence that p300/CBP-mediated acetylation may be a universal and critical modification for p53 function.

Keywords: acetylation/CBP/MDM2/p300/p53

Introduction

The tumor suppressor p53 plays a critical role in human cancer formation. In response to a variety of stress signals, often associated with the progression of neoplastic diseases, p53 becomes activated and induces cell cycle arrest and/or programmed cell death (apoptosis). By eliminating damaged and potentially dangerous cells that might otherwise become cancerous, p53 suppresses tumor formation. In unstressed cells, p53 is latent and is maintained at low levels by targeted degradation mediated by its negative regulator, MDM2 (reviewed in Freedman *et al.*, 1999). The critical role of MDM2 in regulating p53 is best illustrated by a study carried out in mice where inactivation of p53 was shown to completely rescue the embryonic lethality caused by the loss of MDM2 function (Montes de Oca Luna *et al.*, 1995). MDM2 counteracts p53 tumor suppressor activity by physically binding to p53 and suppressing its transcriptional activity. MDM2 also functions as the p53 ubiquitin ligase and triggers its degradation (reviewed in Freedman *et al.*, 1999). This latter activity requires the Ring finger domain located at the C-terminus of MDM2 (Fang *et al.*, 2000), and may also involve the acetyltransferase p300, which binds both MDM2 and p53 (Grossman *et al.*, 1998). Therefore, MDM2 negatively regulates p53 by at least two independent mechanisms.

The activation and stabilization of p53 are thought to be mediated by specific protein modifications, with phosphorylation being the major focus of earlier studies (reviewed in Giaccia and Kastan, 1998; Appella and Anderson, 2000). Although the exact functions of specific phosphorylation events remain controversial, evidence indicates that they probably contribute to both the stabilization and activation of p53. For example, DNA-damaging agents activate phosphorylation at serine (Ser) 15 and Ser37, likely by a family of protein kinases including ATM and ATR (Canman *et al.*, 1998; Tibbetts *et al.*, 1999), and Ser20 by the Chk2 kinase (Hirao *et al.*, 2000; Shieh *et al.*, 2000). These phosphorylation events are believed to contribute to p53 stabilization by preventing the binding of MDM2 and rendering p53 more resistant to MDM2 (Shieh *et al.*, 1997; Unger *et al.*, 1999).

In addition to potentially regulating MDM2 binding, phosphorylation was also shown to modulate the transcriptional activity of p53. For example, phosphorylation at Ser15 stimulates p53 interaction with its transcriptional co-activators p300 and CBP, and a mutation that eliminates this phosphorylation leads to p53 transcriptional defects (Lambert *et al.*, 1998; Dumaz and Meek, 1999). However, the requirement for the aforementioned phosphorylation is probably not universal for p53 stabilization or activation. For example, inhibition of RNA polymerase II by actinomycin D leads to p53 stabilization and activation without invoking either Ser15 or Ser20 phosphorylation (Ashcroft *et al.*, 2000). Similarly, viral oncoprotein E1A-induced p53 activation is not accompanied by Ser15 phosphorylation (de Stanchina *et al.*, 1998). These results suggest that alternative pathways and/or modifications exist and play important roles in modulating p53 activation. One such possible pathway involves the tumor suppressor p19^{ARF}. Inappropriate expression of E1A and other cellular oncogenes, such as c-*myc*, leads to p53 activation through a p19^{ARF}-dependent pathway (de Stanchina *et al.*, 1998; Zindy *et al.*, 1998). p19^{ARF} functions, at least in part, by binding to MDM2 and neutralizing its activity (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998). p19^{ARF} inhibits the p53 ubiquitin ligase activity of MDM2 *in vitro* (Honda and Yasuda, 1999), and sequesters MDM2 into nucleoli,

thereby preventing its nuclear export *in vivo* (Weber *et al.*, 1999). Because the ubiquitin ligase activity and the nuclear export of MDM2 appear to be essential for the degradation of p53 (Tao and Levine, 1999a,b), it is possible that by directly binding and inactivating MDM2, $p19^{ARF}$ bypasses the need for phosphorylation in p53 activation.

Another potential mechanism that may play a critical role in p53 activation is acetylation. Multiple lysine (Lys) residues in p53 are reported to be acetylated. *In vitro*, Lys320 can be acetylated by P/CAF (p300/CBP associated factor) (Liu *et al.*, 1999) and CBP (A.Ito and T.P.Yao, unpublished result), while Lys373 and Lys382 are acetylated by p300 and CBP (Sakaguchi *et al.*, 1998; Liu *et al.*, 1999). At least two additional lysine residues (Lys370 and Lys381) are acetylated by CBP (A.Ito and T.P.Yao, unpublished result). *In vivo* studies show that some of these sites are acetylated in response to DNA-damaging agents, demonstrating that acetylation is a bona fide modification for p53 (Sakaguchi *et al.*, 1998; Liu *et al.*, 1999). However, despite the observation that acetylation can stimulate p53 DNA binding activity *in vitro* (Gu and Roeder, 1997; Liu *et al.*, 1999), the exact function of acetylation and the identities of the p53 acetylases that modify these sites *in vivo* remain to be established.

p300 and its family member CBP are the candidate *in vivo* p53 acetylases. p300 and CBP were originally discovered as transcriptional co-activators that play critical roles in integrating multiple signal-dependent transcription events (reviewed in Goodman and Smolik, 2000). *In vivo*, genetic experiments have clearly demonstrated essential roles for p300 and CBP in normal embryonic development (Tanaka *et al.*, 1997; Yao *et al.*, 1998; Kung *et al.*, 2000). More recent analyses have indicated that p300 and CBP may have specific roles in tumor suppression pathways. p300 mutations were recently found in many types of tumor (Gayther *et al.*, 2000) and mutation of human CBP causes Rubinstein–Taybi syndrome (RTS), which leads to an increased risk of cancers (reviewed in Giles *et al.*, 1998). The human genetic evidence was further substantiated by the analysis of CBP knockout mice, which also display a higher risk of tumors of hematopoietic origin (Gayther *et al.*, 2000; Kung *et al.*, 2000). Interestingly, many of the p300 mutations identified from tumors actually result in the loss of acetyltransferase activity (Gayther *et al.*, 2000), suggesting that the ability of p300 and CBP to acetylate one or more cellular proteins may be critical for their functions in growth control. The fact that p300 and CBP play important roles in p53 transcriptional activity (Gu *et al.*, 1997; Lill *et al.*, 1997) suggests that p53 might be a critical substrate of p300/CBP in mediating tumor suppression.

In this report, we present evidence that acetylation is a common modification associated with p53 activation in response to all p53-activating agents tested. We also establish that, *in vivo*, p300 and CBP can function as p53 acetylases and positively regulate p53 acetylation status, while MDM2 suppresses p53 acetylation. Consistent with p53 acetylation being a critical target of MDM2, we show that the tumor suppressor $p19^{ARF}$ can specifically inhibit the ability of MDM2 to negatively regulate p53 acetylation. Lastly, we provide evidence that inhibition of deacetylation increases the half-life of p53, suggesting that acetylation plays a role in p53 stability. Our results provide strong evidence that acetylation is a tightly regulated event and may be a universal and critical modification for p53 function.

Results

To initially address the potential importance of acetylation, we first determined whether p53 becomes acetylated in response to various environmental or cellular insults that are known to activate and stabilize p53. We used an antibody that specifically recognizes acetylated p53 at Lys382 (Sakaguchi *et al.*, 1998) or an antibody that recognizes a cluster of acetylated lysine residues (pan-acetylated p53, including lysines 370, 372, 373, 381 and 382) to confirm specific acetylation. Because in most cases both antibodies give very similar results in assessing p53 acetylation *in vivo* (for example, see Figure 2), the majority of results in this report are based on the analysis of Lys382 acetylation.

p53 acetylation is commonly induced by multiple p53-activating agents

Consistent with earlier reports, DNA damaging agents, such as UV irradiation (Figure 1A) and the DNA strand breakers camptothecin and *cis*-platinum (data not shown), all efficiently induce p53 acetylation. However, in the earlier reports the deacetylase inhibitor trichostatin A (TSA) was added during treatment to enhance the acetylation signal. This treatment prevents analysis of the kinetics of p53 acetylation (Sakaguchi *et al.*, 1998). To address this issue, we carried out the experiment in the absence of TSA. As shown in Figure 1A, p53 acetylation is a transient event and, after an initial increase, the abundance of acetylated p53 decreased due to the activity of a putative p53 deacetylase. Importantly, the kinetics of p53 acetylation paralleled that of its stabilization, suggesting that acetylation may play a role in p53 activation (Figure 1A).

To investigate further the involvement of acetylation in p53 activation, we examined whether p53-activating agents other than DNA damaging treatment can induce p53 acetylation. Many different types of cellular and environmental insult are capable of activating p53. Here we tested hypoxia, oxidative stress, blocking of nuclear export by leptomycin B (LMB) and depletion of ribonucleotides pools by *n*-phosphonacetyl-L-aspartate (PALA) (reviewed in Giaccia and Kastan, 1998; Freedman *et al.*, 1999). All of these treatments are capable of activating and stabilizing p53. As shown in Figure 1B–E, these agents stabilized p53 and, in every single case, p53 became acetylated. Importantly, treatment with the proteasome inhibitor LLnV, despite its ability to increase total p53 levels, did not result in increased acetylation, demonstrating that the acetylation signals detected were specific and not simply a consequence of higher protein levels (LL in Figure 1B and C). Inhibition of RNA polymerase II by actinomycin D is unique and different from DNA damaging or hypoxia treatment as it activates p53 without triggering phosphorylation of Ser15 or Ser20 (Ashcroft *et al.*, 2000, and data not shown). Figure 1F shows that actinomycin D still efficiently

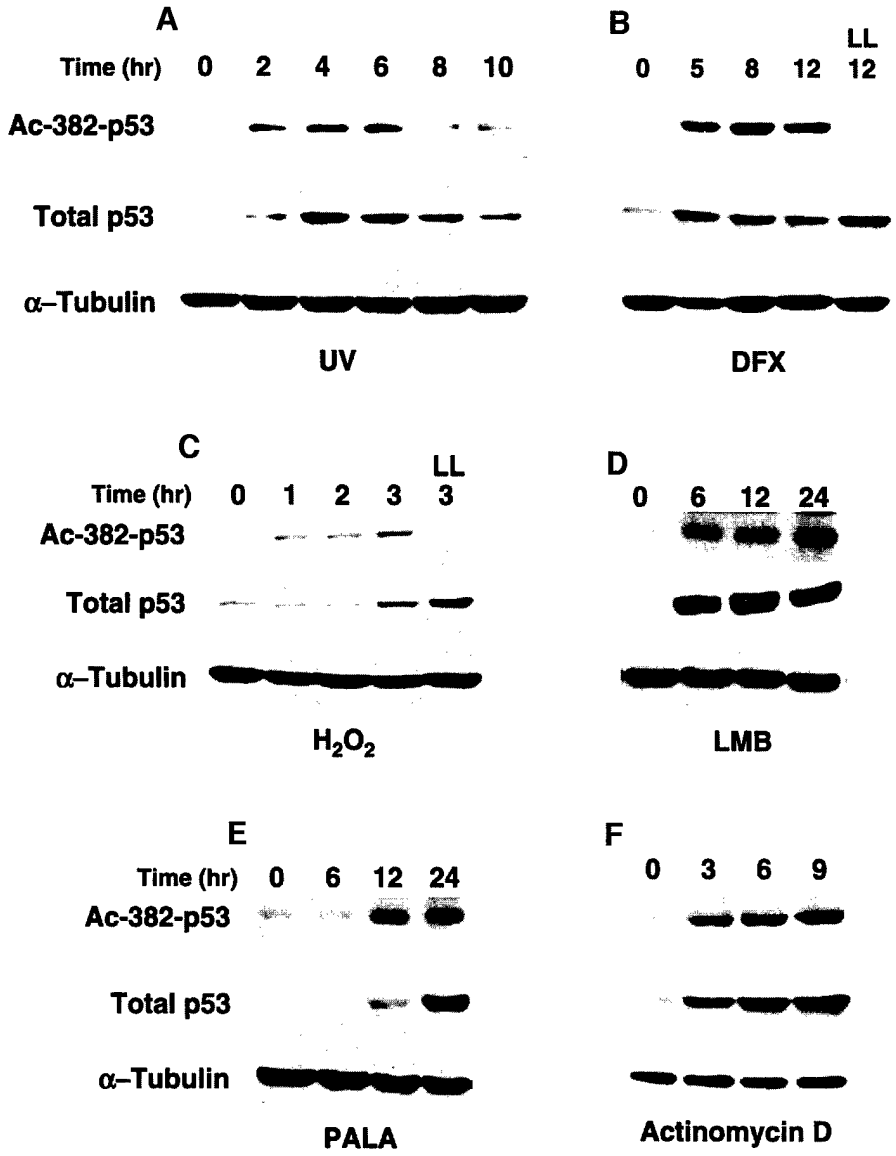

Fig. 1. p53 acetylation induced by multiple p53-activating agents. A549 cells were treated with (**A**) UV-B (100 J/m^2), (**C**) H_2O_2 (1 mM) or proteasome inhibitor LLnV (LL, 10 μM) for 3 h, (**D**) LMB (10 ng/ml) or (**F**) actinomycin D (5 nM). A549 and MCF7 cells (data not shown) were exposed to (**B**) deferoxamine mesylate (DFX) to mimic hypoxia (100 μM) or proteasome inhibitor LLnV (LL, 10 μM) for 12 h. WI-38 cells were exposed to (**E**) PALA (100 μM). (A–F) All cells were harvested at the times indicated. All cells contain wild-type p53. Total p53, acetylated p53 and the internal control α-tubulin levels were assessed by western blotting with α-p53 monoclonal antibody (middle panel), α-acetylated p53 (Lys382) (top panel) and α-tubulin monoclonal antibody (bottom panel), respectively. All treatments were carried out without the use of TSA, except for the DFX experiment where 5 μM of TSA was added to cells.

induced p53 acetylation, distinguishing acetylation from phosphorylation during p53 activation. Altogether, these results demonstrate that p53 becomes acetylated in response to all p53-activating agents tested in this study, and further indicate that acetylation is a common modification associated with p53 activation.

p300 and CBP function as p53 acetylases in vivo

Prime candidates for the p53 acetylases are p300 and its family member CBP. Both p300 and CBP can acetylate p53 *in vitro* (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998; Liu *et al.*, 1999; Figure 4A). However, it is not known whether these acetyltransferases can function as p53 acetylases *in vivo*. To address this issue, we determined whether overexpression of p300 or CBP can induce the specific acetylation of endogenous p53. As shown in Figure 2A, overexpression of wild-type p300 in human 293T cells significantly induced p53 acetylation levels as illustrated by antibodies against acetylated Lys382 (top panel), pan-acetylated p53 (middle panel) or acetylated Lys373 (data not shown). The acetylation of p53 depends on the acetyltransferase activity of p300, as an acetylase-deficient point mutant (DY mutant) derived from a human tumor mutation (C.-H.Lai and T.-P.Yao, manuscript in preparation) failed to induce p53 acetylation. In contrast to p300 or CBP, the expression of P/CAF, which acetylated p53 at Lys320 *in vitro*, did not result in acetylation detectable by the antibodies used in this study (Figure 2A). In p53-null H1299 cells, co-expression of wild-type p53 and p300 also led to specific acetylation of the transfected

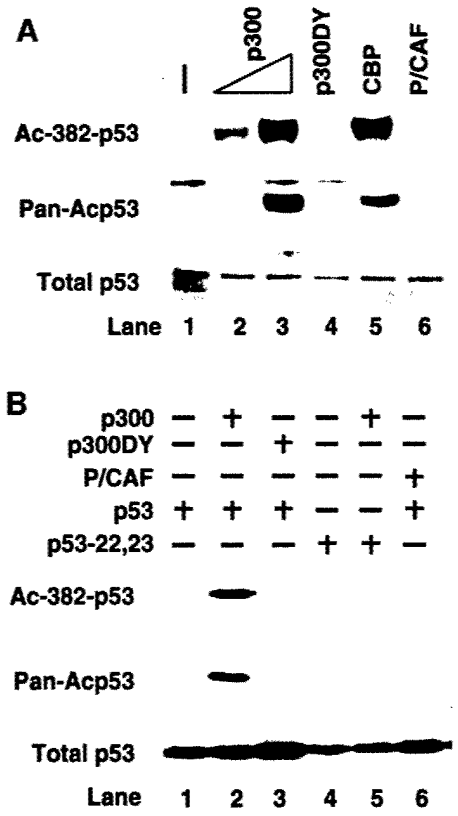

Fig. 2. Acetylation of p53 by p300 and CBP *in vivo*. (**A**) 293T cells were transfected with p300 (lanes 2 and 3), acetyltransferase-deficient p300 DY mutant (lane 4), CBP (lane 5) or PCAF (lane 6), and levels of endogenous acetylated p53 were assessed by either antibody specific for acetylated Lys382 (Ac-382-p53) or antibody against a cluster of acetylated lysines (Pan-Acp53; see text for details). The protein levels of p300, p300 DY, CBP and P/CAF were all comparable (data not shown). (**B**) H1299 cells ($p53^{-/-}$) were transfected with expression plasmid for wild-type p53 alone (lane 1), or co-transfected with either p300 (lane 2), p300 DY mutant (lane 3) or PCAF (lane 6). H1299 cells were also transfected with an expression plasmid for p53(22,23) p300 binding mutant alone (lane 4) or co-transfected with p300 (lane 5). For (A) and (B), cell extracts were prepared (36 h post-transfection) and the detection of acetylated p53 (Ac-382-p53 and Pan-Acp53) or total p53 was determined as described in the legend to Figure 1.

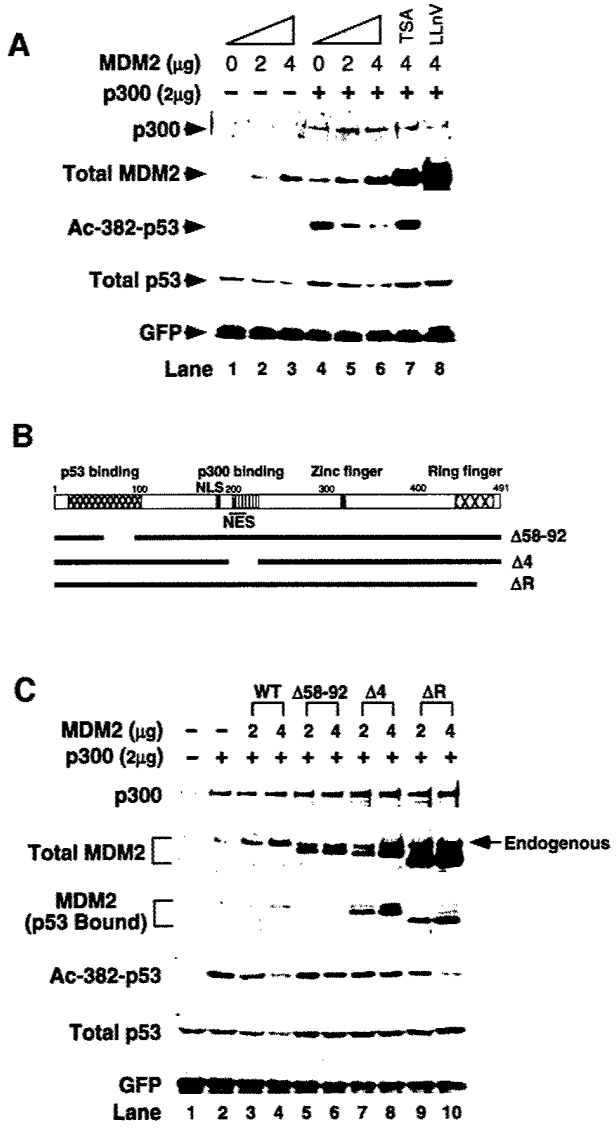

Fig. 3. Suppression of p300-dependent p53 acetylation by MDM2. (**A**) H1299 cells were transfected with expression plasmid for wild-type p53 and internal control GFP (lane 1), and co-transfected with either MDM2 (lanes 2 and 3), c-myc-tagged p300 (lane 4), or MDM2 and c-myc-tagged p300 (lanes 5–8). Cells were also treated 24 h post-transfection with either the deacetylase inhibitor TSA (5 μM) (lane 7) or the proteasome inhibitor LLnV (10 μM) (lane 8) for 12 h. Cell extracts were prepared (36 h post-transfection) and the level of acetylation (third panel) and total p53 protein (fourth panel) were determined by western blotting as described for Figure 1. (**B**) Schematic diagram of MDM2 deletion mutants used in (C). (**C**) H1299 cells were transfected with p53 wild-type and internal control GFP (lane 1), or co-transfected with c-myc-tagged p300 (lane 2), or c-myc-tagged p300 and the indicated amounts of MDM2 wild type (lanes 3 and 4), Δ58–92 mutant (lanes 5 and 6), Δ4 mutant (lanes 7 and 8) or ΔR mutant (lanes 9 and 10). p53 protein and acetylation levels were determined as described in (A). p300 levels were determined by either anti-myc (A14, Santa Cruz) (A) or by anti-p300 (RW128) (C). Both antibodies yielded similar results.

p53 species similar to that observed for the endogenous p53 (Figure 2B, lane 2). Again, the acetylation of the transfected p53 required wild-type p300 acetyltransferase activity (Figure 2B, lane 3). Importantly, a p300-binding-deficient p53 mutant could not be acetylated when co-expressed with p300 (lanes 4–5). This result indicates that direct binding between p53 and p300 is necessary for efficient acetylation and provides further evidence that p53 acetylation is mediated directly by p300 *in vivo*. Identical results were observed when CBP was evaluated for its role in p53 acetylation (Figure 2A, lane 5, and data not shown).

MDM2 suppresses p300/CBP-dependent p53 acetylation in vivo

The results presented thus far provide strong evidence that p53 is acetylated by its positive regulators p300 and CBP in response to a variety of signals. If acetylation plays a critical role in p53 function, it is likely that factors that negatively regulate p53 activity might interfere with this process. MDM2 is the most important p53 negative regulator and it also interacts with p300 (Grossman *et al.*, 1998). These observations prompted us to ask whether MDM2 has the capacity to regulate the acetylation status of p53. As shown in Figure 3A, overexpression of MDM2 effectively reduced p300-dependent p53 acetylation in a

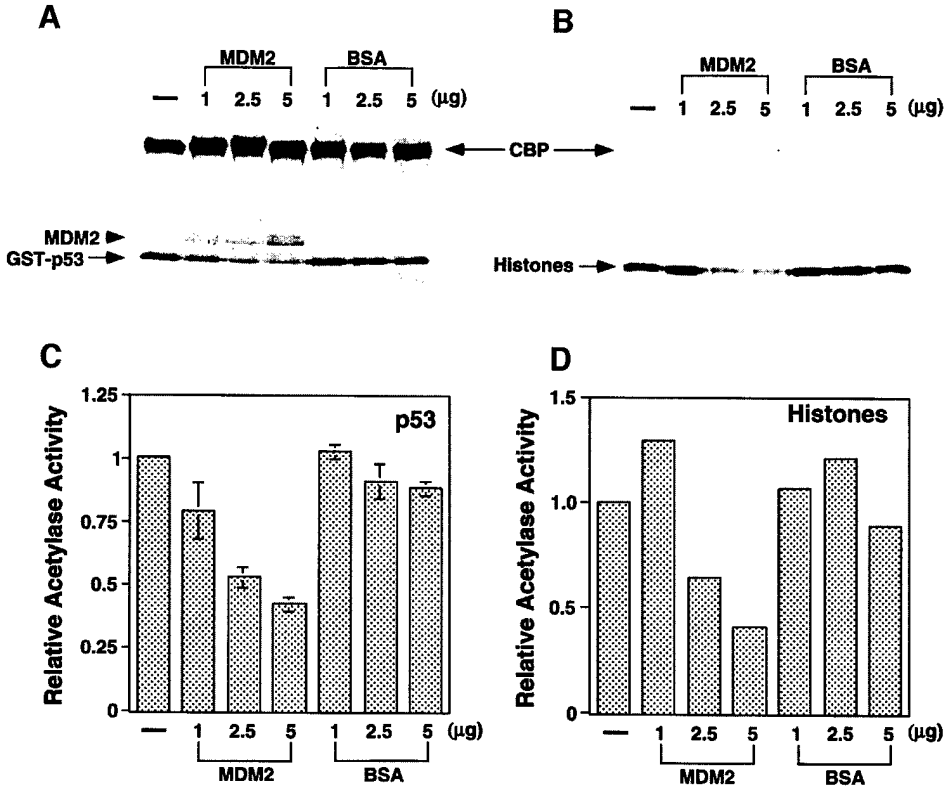

Fig. 4. Suppression of CBP acetyltransferase activity by MDM2 *in vitro*. (A and B) GST–p53 (**A**) or core histones (**B**) were acetylated by recombinant CBP in the presence of the indicated amounts of MDM2 or BSA, and analyzed by SDS–PAGE followed by autoradiography. Film was exposed (A) overnight and (B) for 3 h. Acetylated p53 and histone are indicated with arrows. Note that the level of acetylated p53 and acetylated histone decreases in the presence of MDM2. Acetylated MDM2 is marked with an arrowhead (A). (C and D) The intensity of the acetylated GST–p53 (**C**) or histones (**D**) was quantified by phosphoimager analysis and plotted. The intensity of acetylated GST–p53 or histones in the absence of MDM2 or BSA was set as 1. (C) reflects the average of three experiments, while (D) reflects the average of two experiments.

dose-dependent manner (lanes 4–6). Of note, MDM2 overexpression does not affect the protein levels of transfected p300 (Figure 3A, top panel), supporting a direct effect of MDM2 on p53 acetylation. To rule out the possibility that the decrease in acetylation was caused by a corresponding decrease in p53 protein levels triggered by MDM2, the proteasome inhibitor LLnV was added to the culture to block p53 degradation. This treatment led to the stabilization of p53. Despite high protein levels, p53 remained non-acetylated in the presence of MDM2 (Figure 3A, lane 8). This result demonstrates that MDM2 can reverse the p53 acetylation induced by p300. In contrast to LLnV treatment, the deacetylase inhibitor TSA effectively abrogated the effect of MDM2 and restored p53 acetylation (Figure 3A, compare lanes 6 and 7), providing further evidence that MDM2 specifically modulated p53 acetylation. Interestingly, TSA treatment also increased p53 protein levels, suggesting the possibility that inhibition of p53 deacetylation promoted p53 stability (see below).

To study further how MDM2 suppresses p53 acetylation, we analyzed a series of MDM2 mutants with specific functional domains deleted (Figure 3B). Specifically, we tested MDM2 mutants that are deficient in p53 binding (Δ58–92) (Chen *et al.*, 1993), p300 binding (Δ4, amino acids 192–222) (Grossman *et al.*, 1998), or defective in ubiquitin ligase activity (ΔR, deletion of the Ring domain). As shown in Figure 3C, after transfection into H1299 cells, all these MDM2 variants were expressed (second panel). However, when compared with wild-type MDM2 (lanes 3–4), both the p53 binding mutant (Δ58–92, lanes 5–6) and the p300 binding mutant (Δ4, lanes 7–8) were defective as they only weakly suppressed p53 acetylation even when expressed at higher levels (Figure 3C, Ac-382). Importantly, both mutants are also deficient in degrading p53, further suggesting a functional link between p53 acetylation and stability. In contrast, the Ring domain mutant inhibited p53 acetylation to a level similar to that of wild-type MDM2 (ΔR, lanes 9–10). These results indicate that physical binding to both p53 and p300 is required for full activity of MDM2 to repress p53 acetylation. The Ring domain, which is essential for degrading p53 (Fang *et al.*, 2000), is dispensable for this function. From this set of experiments, we conclude that MDM2 can actively repress p300-mediated p53 acetylation *in vivo* and that this activity requires physical binding to both p53 and p300.

MDM2 suppresses CBP acetyltransferase activity in vitro

In principle, MDM2 could repress p53 acetylation either by directly suppressing p53 acetylation or by promoting p53 deacetylation. To address these possibilities, we first determined whether MDM2 could directly inhibit

p300/CBP-mediated p53 acetylation *in vitro*. As shown in Figure 4A, although recombinant MDM2 had no effect on CBP auto-acetylation, it efficiently inhibited p53 acetylation in a dose-dependent manner (Figure 4A and C). As this inhibition was not sensitive to the deacetylase inhibitor TSA (data not shown), MDM2 likely interfered with the acetyltransferase activity of CBP rather than functioning as a p53 deacetylase. Interestingly, while suppressing p53 acetylation, MDM2 itself became acetylated by CBP (Figure 4A, arrowhead). The functional importance of this acetylation is not yet clear. To determine whether the effect of MDM2 on CBP was specific to p53 acetylation, we tested whether MDM2 could suppress CBP-mediated histone acetylation. As shown in Figure 4B and D, MDM2 was able to suppress the acetylase activity of CBP towards core histones as well. In contrast, under the same experimental conditions, MDM2 had no apparent suppressive effect on another acetyltransferase, P/CAF (data not shown). Thus, MDM2 can specifically suppress CBP-mediated p53 and core histone acetylation *in vitro*. These observations suggest that the ability of MDM2 to repress p53 acetylation *in vivo* works, at least in part, by suppressing the acetyltransferase activity of p300 and CBP.

Tumor suppressor p19^{ARF} reverses the inhibition of p53 acetylation by MDM2

p19^{ARF} induces p53 activation by negatively regulating MDM2. This activity is proposed to be mediated by inactivating p53 E3 ligase activity of MDM2. Analysis of the role of MDM2 in p53 acetylation suggests an alternative possibility that p19^{ARF} might function by antagonizing the activity of MDM2 toward p53 acetylation. To examine this possibility, we determined whether co-expression of p19^{ARF} and MDM2 could neutralize the latter's ability to repress p300-dependent p53 acetylation. As shown in Figure 5, in the absence of p19^{ARF}, co-transfection of MDM2 efficiently repressed p53 acetylation and induced its degradation in H1299 cells (compare lanes 2 and 3). However, upon co-expression of p19^{ARF}, both p53 acetylation and protein levels were restored (lanes 4–5). Importantly, a p19^{ARF} mutant, which does not bind MDM2 (C65, Zhang and Xiong, 1999) failed to suppress MDM2 in this assay (lane 6). Altogether, these results demonstrate that p19^{ARF} can abrogate the ability of MDM2 to suppress p53 acetylation. The correlation between p53 protein level and acetylation level in response to MDM2 and p19^{ARF}, however, does suggest that acetylation might influence p53 stability.

An increased level of total and p53-bound MDM2 was also observed when p19^{ARF} was co-expressed (Figure 5, MDM2 panels, lanes 4–5). This might be due to the inhibition of MDM2 auto-ubiquitylation (Fang *et al.*, 2000) and, consequently, the stabilization of MDM2. Importantly, despite the high levels of MDM2 associated with p53, MDM2 in this complex did not show appreciable repression toward p53 acetylation, supporting the idea that p19^{ARF} dominantly inhibits the activity of MDM2 in this assay. This observation also suggests that p19^{ARF} restores p53 acetylation and protein levels without dissociating MDM2 from p53. This set of results demonstrates that, in addition to inhibiting MDM2 as a p53 ubiquitin ligase, p19^{ARF} is also capable of inactivating MDM2 in suppressing p53 acetylation, providing further evidence that acetylation is a modification regulated by a p300/CBP–MDM2–p19^{ARF} feedback loop in the p53 network.

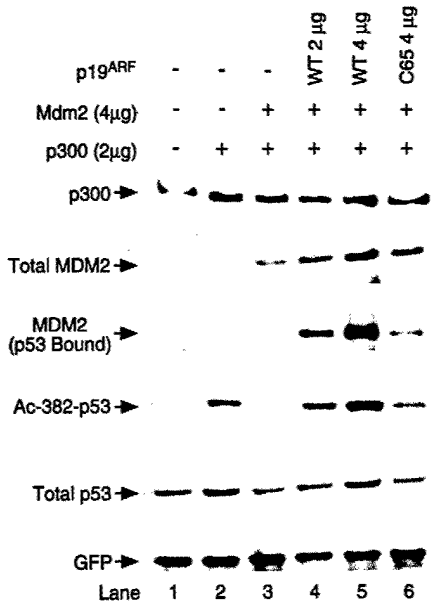

Fig. 5. p19^{ARF} reverses the inhibition of p53 acetylation by MDM2. H1229 cells were transfected with either expression plasmid for p53 wild-type and internal control GFP (lane 1) or co-transfected with c-myc-tagged p300 (lanes 2–6), in combination with MDM2 and p19 expression plasmid as indicated. Analysis of p53 protein and acetylation levels was carried out as described for Figures 1 and 2. Note that expression of p19 effectively neutralized the effects of MDM2 on p53 acetylation (lanes 4 and 5). p300 levels were determined by RW128.

Inhibition of p53 deacetylation promotes p53 stability

The results presented so far support the idea that acetylation is a common modification regulated by a network of critical regulators of p53 function. In principle, acetylation could contribute to p53 stabilization and/or p53 activity. Several observations from our study suggest the possibility that acetylation may regulate p53 stability. First, there was a positive correlation between the kinetics of p53 protein levels and its acetylation levels in response to DNA damage (Figure 1A). Secondly, p19^{ARF} concomitantly restored p53 protein and acetylation levels, which were negatively regulated by MDM2 (Figures 3 and 5). Lastly, treatment with the deacetylase inhibitor TSA seemed to result in higher p53 protein levels (Figure 3A). If acetylation were important for p53 stabilization, one would predict that TSA treatment should delay the normal rate of degradation by preventing p53 deacetylation. To test this hypothesis, p53 stability was determined following its activation by UV irradiation. As shown in Figure 6A (top panel), TSA treatment effectively inhibited the p53 deacetylase and increased the levels of acetylated p53 in A549 cells. Importantly, the apparent half-life of p53 was dramatically increased in the presence of TSA, suggesting that acetylated p53 is more stable (Figure 6A, middle panel, and B). In contrast, the same treatment did not affect the half-life of actin (Figure 6A, bottom panel), indicating that TSA did not have a general positive effect on protein

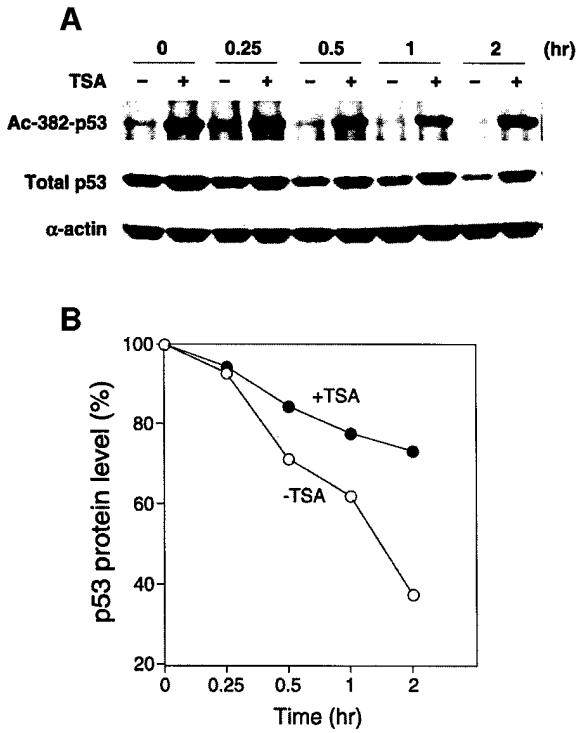

Fig. 6. Inhibition of deacetylase promotes p53 stabilization. (**A**) A549 cells were exposed to UV-B (50 J/m^2) in the presence (+) or absence (–) of TSA (5 μM). Four hours post-irradiation, cyclohexamide (10 μg/ml) was added to inhibit new p53 protein synthesis (designated 0 h). Cells were harvested at the time-points indicated after cyclohexamide treatment. Acetylated p53 (top panel) and total p53 (middle panel) were determined. Note that total p53 level and acetylation levels are significantly higher in the presence of TSA. As a control, direct western blotting with an α-actin polyclonal antibody also assessed actin levels (lower panel). (**B**) The band intensity of p53 protein levels was measured by NIH imaging software and calculated against the amount of p53 present at time point 0, which was set at 100%. Results are given in the presence (filled circles) or absence (empty circles) of TSA treatment.

stability. This result indicates that one function of specific p53 acetylation is to increase its stability.

Discussion

In this report, we show that p53 becomes acetylated in response to all p53-activating agents tested (Figure 1). Together with the recent report that p53 acetylation increases as fibroblasts senesce (Pearson *et al.*, 2000), these results clearly establish acetylation as a common modification that invariably accompanies p53 activation. This is in contrast to the two well studied phosphorylation events on Ser15 and Ser20, which are activated only by a select few agents (Ashcroft *et al.*, 2000), and further illustrates a unique requirement for acetylation in p53 activation. Although the complete function of p53 acetylation remains to be firmly established, we provide evidence that acetylation may at least contribute to p53 stability. Two recent reports have suggested that acetylation is important for p53 to suppress oncogenic ras-induced transformation (Pearson *et al.*, 2000) and to induce metaphase chromosome fragility (Yu *et al.*, 2000), adding more evidence for the functional significance of p53 acetylation. The findings that p300/CBP acetyltransferases and p19^{ARF} promote p53 acetylation *in vivo*, while MDM2 inhibits acetylation, lend support to the idea that acetylation is an important modification targeted by both positive and negative regulators critical to p53 tumor suppressor activity.

Reversible acetylation was originally identified in histones and was thought to be important for transcriptional activity (Wade *et al.*, 1997). However, a growing number of non-histone proteins are now being reported as targets of acetylation (reviewed in Kouzarides, 2000). Although in most cases the function of acetylation remains to be firmly established, analysis of E2F1 and myoD indicates that P/CAF-mediated acetylation appears to increase E2F1 stability (Martinez-Balbas *et al.*, 2000) and contribute to myoD activity (Sartorelli *et al.*, 1999). Similarly, p300/CBP-dependent GATA-1 acetylation has been shown to be critical for GATA-1 function (Boyes *et al.*, 1998). In this report, we further show that MDM2 may be an acetylated protein as well (Figure 4A). Together with the demonstration that acetylation of p53 is tightly regulated and is important for p53 stability, these various lines of evidence support the hypothesis that acetylation is a prominent and likely general regulatory modification used to modulate protein function.

We have presented evidence that p300 and CBP are able to acetylate p53 and are likely to be the key p53 acetylases *in vivo*. Biochemical and genetic experiments indicate that p300 and CBP levels are limited in cells (reviewed in Goodman and Smolik, 2000), and apparently, they cannot support endogenous p53 acetylation under normal conditions. In theory, high levels of p300/CBP in the transfection setting should increase the probability of complex formation with p53. Moreover, transfection itself probably triggers some DNA damage response. These two factors together may contribute to p53 acetylation upon p300/CBP overexpression (Figure 2). Consistent with this idea, we have shown that a direct interaction between p300/CBP and p53 is necessary for efficient p53 acetylation (Figure 2B). Under normal physiological settings, it is likely that p53 and p300/CBP complexes are induced in response to activating signals. Consistent with this idea, we have found that mutations that eliminate phosphorylation at Ser15 but not Ser20 significantly reduced p53 acetylation *in vivo* (our unpublished observation). As Ser15 phosphorylation stimulates p53 binding to p300/CBP (Lambert *et al.*, 1998), this result provides evidence that specific phosphorylation on Ser15 could be one activation step leading to p53–p300/CBP complex formation and subsequent p53 acetylation by p300/CBP. Ser15 phosphorylation, however, is not the only mechanism that can lead to p53 acetylation. Actinomycin D does not induce Ser15 phosphorylation (Ashcroft *et al.*, 2000), yet it is a powerful agent in triggering p53 acetylation (Figure 1F). This result suggests a more general and unique requirement for acetylation than some specific phosphorylation events during p53 activation. The mechanism by which actinomycin D induces p53 acetylation without Ser15 phosphorylation, however, remains unknown.

Our results show clearly that MDM2 can suppress p300/CBP-mediated p53 acetylation *in vitro* and *in vivo*. There are at least four possible mechanisms that may explain this

observation. First, MDM2 binds and inactivates p300/CBP acetyltransferase activity. This possibility is supported by our result that a p300-binding-deficient MDM2 mutant is defective in this activity. Secondly, p300/CBP and MDM2 bind to non-identical but overlapping regions at the N-terminus of p53. It is possible that high levels of MDM2 bind p53 and displace p300/CBP, thereby inhibiting p53 acetylation. This mechanism, however, may not explain how MDM2 suppresses histone acetylation, as there is no evidence that MDM2 binds histones. The observation that $p19^{ARF}$ restores p53 acetylation without dissociating MDM2 from p53 is also inconsistent with this model (Figure 5). Thirdly, MDM2 can interact directly with p300/CBP and itself becomes acetylated (Figure 4A). It is possible that MDM2 serves as a substrate competitor and thereby suppresses p53 acetylation. Further studies will be needed to verify the acetylation of MDM2 *in vivo* and the importance of this acetylation. Lastly, although MDM2 inhibits p53 acetylation by CBP directly *in vitro*, we could not eliminate the possibility that other mechanisms may also contribute to the suppression of p53 acetylation *in vivo*. For instance, MDM2 could stimulate deacetylation by recruiting a p53 deacetylase. The observation that TSA can completely abrogate the inhibitory effect of MDM2 on p53 acetylation (Figure 3A) and that MDM2 interacts with a specific deacetylase (A.Ito and T.P.Yao, unpublished result) is consistent with this possibility. Regardless of which mechanism is correct, our results clearly demonstrate that MDM2 is able to suppress p53 acetylation *in vivo* and *in vitro*.

Our analysis of MDM2 also reveals that MDM2 suppresses the core histone acetylation induced by p300/CBP. It has been hypothesized that p300 and CBP activate transcription by acetylating histones. The inhibitory activity of MDM2 on histone acetylation provides a biochemical mechanism to explain how MDM2 can inhibit p53 transactivation potency. In this scenario, the recruitment of MDM2 to the p53–p300 or p53–CBP complexes on target chromatin inhibits histone acetylation and thereby represses p53-dependent transcription.

By binding to MDM2, $p19^{ARF}$ plays a critical role in p53 activation. This activity of $p19^{ARF}$ was attributed, at least in part, to its ability to suppress the MDM2 E3 ligase activity toward p53 ubiquitylation (Honda and Yasuda, 1999). Our study now shows that $p19^{ARF}$ can also abrogate the inhibitory effect of MDM2 toward p53 acetylation *in vivo* (Figure 5). In fact, overexpression of $p19^{ARF}$ alone is sufficient to induce p53 acetylation (A.Ito, unpublished result). This observation adds a novel mechanism through which $p19^{ARF}$ regulates MDM2 activity and participates in tumor suppression. Two alternative hypotheses have been put forward to explain how $p19^{ARF}$ inhibits MDM2 activity. One proposes that $p19^{ARF}$ sequesters MDM2 in nucleoli and dissociates MDM2 from p53 (Weber *et al.*, 1999), while the other shows that p53–MDM2–$p19^{ARF}$ forms a tripartite complex in the nucleoplasm, where MDM2 is not active (Zhang and Xiong, 1999). Unexpectedly, we found that upon $p19^{ARF}$ expression, a dramatic increase in MDM2 was found to complex with p53. However, the MDM2 in this complex is not active in suppressing p53 acetylation (Figure 5, lanes 4 and 5). These observations are more consistent with the possibility of a tripartite complex formation wherein $p19^{ARF}$ dominantly inhibits the activity of MDM2 toward p53 acetylation. However, we have found that recombinant $p19^{ARF}$ does not interfere with the ability of MDM2 to suppress CBP-mediated p53 acetylation *in vitro* (A.Ito and T.P.Yao, unpublished observation). Further studies will be required to determine how $p19^{ARF}$ suppresses MDM2 activity in this ternary complex. Regardless, our study demonstrates that $p19^{ARF}$, in addition to regulating MDM2 ubiquitin ligase activity, can also suppress the activity of MDM2 towards p53 acetylation. These results suggest that all major regulators of p53 activity, including p300/CBP, MDM2 and $p19^{ARF}$, integrate different extracellular and intracellular signals to modulate p53 acetylation level and thereby its stability and activity.

What is the importance of p53 acetylation in relation to p53 function? It was first reported that acetylation increases p53 DNA binding activity *in vitro* (Gu and Roeder, 1997). However, analyses of p53 mutants that can not be acetylated do not reveal obvious defects in DNA binding *in vivo* (data not shown), suggesting that acetylation might have other functions. Three lines of evidence derived from this study suggest that acetylation functions, at least in part, by modulating p53 stability. First, there is positive correlation between endogenous p53 protein and acetylation levels upon normal p53 activation (Figure 1). Secondly, in analyzing the ability of various MDM2 mutants and $p19^{ARF}$ to regulate p53 acetylation (Figures 3 and 5), we found a similar correlation between p53 protein and acetylation levels. This conclusion is further supported by the observation that TSA can efficiently reverse the degradation of p53 induced by MDM2 (Figure 3A and data not shown). Thirdly, prevention of p53 deacetylation leads to a more stable p53 species (Figure 6). Given that acetylation is always accompanied by p53 stabilization (Figure 1), this correlative evidence strongly suggests that acetylation may be a modification that contributes to p53 stabilization. How does acetylation stabilize p53? Since both the acetyltransferase and ubiquitin-conjugating system through which p53 is targeted for degradation modify lysine, it is possible that acetylation protects lysine residues from being ubiquitylated. It was reported recently that several lysine residues located at the C-terminus target p53 for ubiquitylation and degradation (Rodriguez *et al.*, 2000). Importantly, these are the same lysine residues that can be acetylated by p300/CBP. These observations suggest the possibility that acetylation renders lysines unavailable for the ubiquitin-conjugating machinery, and thereby promotes p53 stability. If acetylation functions, at least in part, to inhibit ubiquitylation, reversible acetylation might have a more general role in regulating protein stability.

In conclusion, we propose that in response to cellular stresses, p53 becomes acetylated by the p300/CBP acetyltransferases. This modification requires either specific phosphorylation, such as at Ser15, or the activation of tumor suppressor $p19^{ARF}$. Acetylation leads to p53 stabilization and the subsequent induction of MDM2. MDM2 then in turn triggers p53 deacetylation followed by p53 inactivation and destruction. The modulation of p53 acetylation by CBP/p300, MDM2 and $p19^{ARF}$ suggests the existence of an intricate pathway regulating the acetylation equilibrium that is crucial to the tumor suppressor activity

of p53. Further characterization of the function of p53 acetylation will be critical for understanding the regulation of p53 tumor suppressor activity.

Materials and methods

Cell lines and transfection

A549, WI38, 293T and H1299 human cells were maintained in Dulbecco's modified Eagle's medium (DMEM). All cells were grown at 37°C in the presence of 10% fetal bovine serum and penicillin/streptomycin in a humidified atmosphere of 5% CO_2. A549 and WI38 cells have wild-type p53, while H1299 cells are devoid of any p53 expression. All transfections were performed by the calcium phosphate method as described previously (Yao *et al.*, 1992).

Plasmids

Wild-type human p53 cDNA was cloned into the *Bam*HI–*Xho*I site of pCDNA3. The mutant p53(22,33), which can not bind to p300 has been described previously (Gu *et al.*, 1997). The human MDM2 wild-type cDNA was cloned into the *Bam*HI–*Eco*RI site of pCDNA3. The MDM2 ΔR mutant cDNA was made by digesting wild-type pCDNA3-MDM2 with *Sal*I to delete the Ring domain (amino acids 442–491). The MDM2 Δ4 and Δ58–92 mutants were described previously (Chen *et al.*, 1993; Grossman *et al.*, 1998). The human p300-DY (Lys1399 converted to tyrosine) mutant was generated by site-direct mutagenesis and cloned into the pCMV vector. The full length mouse $p19^{ARF}$ and $p19^{ARF}$ N-terminal fragment (C65) (MDM2-binding-deficient mutant) were described previously (Zhang and Xiong, 1999).

Pulse–chase

A549 cells at 80–90% confluence were exposed to a 310 nm wavelength UV source. The deacetylase inhibitor TSA (Sigma) was added at a final concentration of 5 μM immediately after UV irradiation. Four hours after irradiation, cells were treated with 10 μg/ml of cyclohexamide to stop new p53 protein synthesis, and cells were then harvested at the indicated time points as described in Figure 6.

Immunoprecipitation and immunoblotting

Cells were lysed in buffer [20 mM Tris–HCl pH 7.6, 170 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM dithiothreitol (DTT)] supplemented with 5 μM TSA and protease inhibitors. For immunoprecipitation with anti-p53 antibody, equal amounts of lysate (containing 200–300 μg of total cellular protein) were incubated with 1 μg of goat anti-p53 antibody (Santa Cruz) and protein G–Sepharose (Pharmacia) for 3 h at 4°C. The use of goat antibody eliminates the heavy chain signal that co-migrates with p53 in subsequent immunoblotting. For immunoprecipitation with anti-p300 antibody, equal amounts of lysate (containing 100–150 μg of total cellular protein) were incubated with anti-p300 antibody (RW128) and protein G–Sepharose (Pharmacia) for 3 h at 4°C. When immunoprecipitation was not performed, 20–30 μg of total extracts were analyzed. Proteins were detected by chemiluminescent ECL kit (Amersham) with one of the following antibodies: anti-human p53 antibody (Ab-6, Calbiochem), anti-human acetylated (Lys382) p53 antibody (Calbiochem), anti-human MDM2 antibody (SMP14, Santa Cruz), anti-α-tubulin antibody (DM1A, Sigma), anti-c-myc antibody (A14, Santa Cruz), anti-p300 antibody (RW128, Eckner *et al.*, 1994), anti-green fluorescent protein (GFP) antibody (Boehringer Mannheim) or anti-actin antibody (C-11, Santa Cruz).

In vitro acetyltransferase assay

Recombinant CBP protein (1 μg) purified from baculovirus was pre-incubated with the indicated amounts of purified bacterially expressed MDM2 protein or bovine serum albumin (BSA) for 10 min at room temperature. After pre-incubation, substrates [1 μg of glutathione-*S*-transferase (GST)–p53 or histone] were added and incubated with 50 nCi [^{14}C]acetyl-coenzyme A in 30 μl of reaction buffer (50 mM Tris–HCl pH 8.0, 10% glycerol, 1 mM DTT, 100 μM EDTA, 1 mM phenylmethylsulfonyl fluoride) for another 45 min at 37°C. Acetylation was analyzed by SDS–PAGE followed by autoradiography, or by a phosphoimager.

Acknowledgements

We thank Dr W.Gu for his generous gift of the pan-acetylated p53-specific antibody and for sharing unpublished results, Dr S.Grossman for the MDM2 and p53 reagents, Drs E.Hura, J.Nevins and Y.Xiong for the $p19^{ARF}$ reagents and Dr J.Direnzo for CBP baculovirus. We thank Dr K.Sakaguchi for advice on the acetylated p53-specific antibody. We are grateful to Drs C.Anderson, A.R.Means, B.Harvat, Ms C.Hubbert, A.Guardiola and Mr T.Bolger for critically reading the manuscript. M.H. is supported by DAMD17-98-1-8072 and DK50494 from the NIH to Dr D.McDonnell. This work is supported partially by funding from the Damon Runyon–Walter Wichell Cancer Foundation to T.-P.Y. (DRS 20) and the National Institutes of Health (CA85676-01A1).

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Received October 2, 2000; revised January 22, 2001; accepted January 29, 2001

The EMBO Journal Vol. 21 No. 22 pp. 6236–6245, 2002

MDM2–HDAC1-mediated deacetylation of p53 is required for its degradation

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The tumor suppressor p53 is stabilized and activated in response to cellular stress through post-translational modifications including acetylation. p300/CBP-mediated acetylation of p53 is negatively regulated by MDM2. Here we show that MDM2 can promote p53 deacetylation by recruiting a complex containing HDAC1. The HDAC1 complex binds MDM2 in a p53-independent manner and deacetylates p53 at all known acetylated lysines *in vivo*. Ectopic expression of a dominant-negative HDAC1 mutant restores p53 acetylation in the presence of MDM2, whereas wild-type HDAC1 and MDM2 deacetylate p53 synergistically. Fibroblasts overexpressing a dominant negative HDAC1 mutant display enhanced DNA damage-induced p53 acetylation, increased levels of p53 and a more pronounced induction of p21 and MDM2. These results indicate that acetylation promotes p53 stability and function. As the acetylated p53 lysine residues overlap with those that are ubiquitylated, our results suggest that one major function of p53 acetylation is to promote p53 stability by preventing MDM2-dependent ubiquitylation, while recruitment of HDAC1 by MDM2 promotes p53 degradation by removing these acetyl groups.

Keywords: acetylation/HDAC1/MDM2/p53/ubiquitylation

Introduction

The activation of the tumor suppressor p53, which triggers growth arrest and apoptosis in cells that are in danger of becoming cancerous, involves the regulation of p53 stability. In unstressed cells, p53 is maintained at low levels by its key negative regulator MDM2 (Freedman *et al.*, 1999). Although p53 is required for active tumor suppression, the negative regulation of p53 by MDM2 is fundamentally important. This point is best illustrated by a genetic study in mice, where the loss of MDM2 led to early embryonic lethality due to uncontrolled p53 levels and activity (Montes de Oca Luna *et al.*, 1995). The importance of this negative regulation is further supported by the observation that expression of MDM2 is positively regulated by p53 (Wu *et al.*, 1993), and thus establishes a tight negative feedback loop. MDM2 is an E3 ligase that ubiquitylates a defined set of lysine residues at the C-terminus of p53 (Honda *et al.*, 1997; Nakamura *et al.*, 2000; Rodriguez *et al.*, 2000). The MDM2-mediated ubiquitylation of p53 is believed to trigger rapid degradation of p53 by proteasomes (Freedman *et al.*, 1999), or to promote its nuclear export (Boyd *et al.*, 2000; Geyer *et al.*, 2000). Thus, MDM2 functions as a key negative regulator for p53, at least in part, by controlling the p53 ubiquitylation status (Zhang and Xiong, 2001).

p53 is transiently stabilized and activated in response to various cellular insults. The stabilization and activation of p53 are thought to be mediated by post-translational modification events, such as phosphorylation (Giaccia and Kastan, 1998; Appella and Anderson, 2001), which was proposed to interfere with the ability of MDM2 to negatively regulate p53 (Shieh *et al.*, 1997; Unger *et al.*, 1999). Although multiple serine residues are phosphorylated in response to genotoxic stress (Shieh *et al.*, 1997; Canman *et al.*, 1998; Hirao *et al.*, 2000), p53 activation is not always accompanied by the specific phosphorylation events proposed to be important for its function (Ashcroft *et al.*, 2000). In contrast to phosphorylation, we have reported that p53 invariably becomes acetylated in response to a wide variety of cellular insults that are known to be potent p53-activating agents (Ito *et al.*, 2001). Importantly, stress-induced p53 acetylation is transient and reversible, suggesting the existence of negative regulators that keep p53 acetylation in check (Ito *et al.*, 2001). Supporting this hypothesis, we and others have found that MDM2 negatively regulates p53 acetylation (Kobet *et al.*, 2000; Ito *et al.*, 2001). This result suggests a functional link between MDM2, an E3 ubiquitin ligase, and acetylation. The importance of this observation is further supported by the finding that the inhibitory effect of MDM2 on p53 acetylation can be reversed by the tumor suppressor $p14^{ARF}$ (Ito *et al.*, 2001), which binds to MDM2 and inhibits its activity (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998b; Honda and Yasuda, 1999). Together, these observations support the notion that the regulation of acetylation is a central target of the p53–MDM2–$p14^{ARF}$ feedback loop. However, the mechanism by which MDM2 controls p53 acetylation is not completely understood. It was previously shown that MDM2 can suppress CREB binding protein (CBP) acetyltransferase activity *in vitro*. This result supports the idea that MDM2 inhibits p53 acetylation, at least in part, by binding and reducing p300/CBP acetyltransferase activity (Kobet *et al.*, 2000; Ito *et al.*, 2001). However, the observation that the deacetylase inhibitor TSA can restore p53 acetylation in the presence of MDM2 suggests a more complex mechanism, wherein MDM2 might also utilize a deacetylase pathway to control p53 acetylation level (Ito *et al.*, 2001). Uncovering this pathway will be essential to understand-

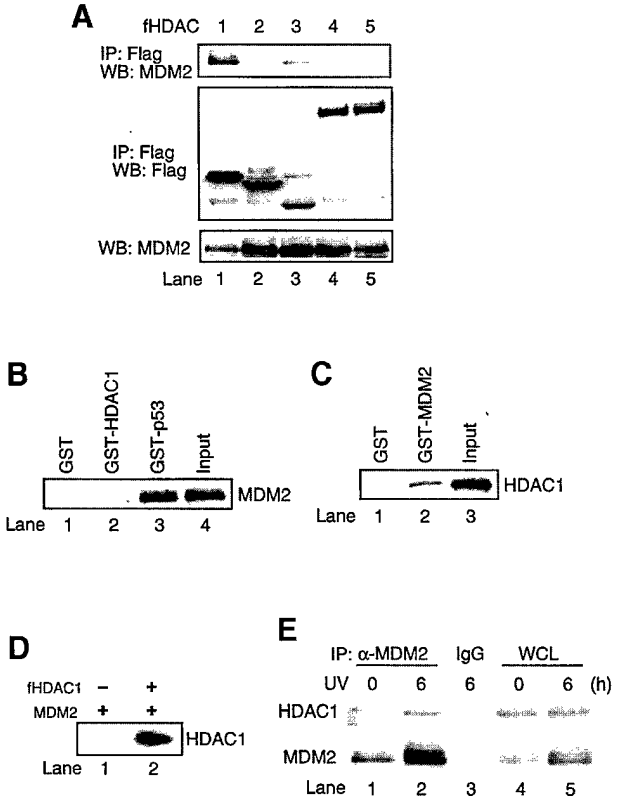

Fig. 1. Interaction between MDM2 and HDAC1. (**A**) 293T cells were cotransfected with 10 μg of MDM2 and 5–10 μg of either Flag-tagged HDAC1 (lane 1), HDAC2 (lane 2), HDAC3 (lane 3), HDAC4 (lane 4) or HDAC5 (lane 5). Cellular extracts were immunoprecipitated with anti-Flag antibody followed by immunoblotting with anti-MDM2 antibody (top panel) or anti-Flag antibody (middle panel). Total MDM2 protein was detected with anti-MDM2 antibody (bottom panel). (**B**) Either GST (lane 1), GST–HDAC1 (lane 2) or GST–p53 (lane 3) were incubated with recombinant MDM2 protein followed by immunoblotting with anti-MDM2 antibody. (**C**) H1299 cells were transfected with Flag-tagged HDAC1 and the cellular extracts were incubated with either GST (lane 1) or GST–MDM2 (lane 2) followed by immunoblotting with anti-Flag antibody. (**D**) H1299 cells were transfected with MDM2 alone (lane 1) or cotransfected with Flag-tagged HDAC1 (lane 2) and cellular extracts were immunoprecipitated with anti-MDM2 antibody followed by immunoblotting with anti-Flag antibody. (**E**) A549 cells were exposed to UV-B irradiation (75 J/m^2) for 6 h and cellular extracts were immunoprecipitated with either anti-MDM2 antibody (lanes 1 and 2) or mouse IgG as a control (lane 3) followed by immunoblotting with anti-HDAC1 antibody (top panel) or anti-MDM2 antibody (bottom panel). Total HDAC1 and MDM2 protein were detected with either anti-HDAC1 antibody (top panel, lanes 4 and 5) or anti-MDM2 antibody (bottom panel, lanes 4 and 5).

ing how p53 acetylation is regulated and is the subject of this study.

The acetylation of p53 is mainly catalyzed by the acetyltransferases p300 and CBP *in vivo* (Ito *et al.*, 2001). Interestingly, acetylation occurs at multiple lysine residues (six in total, see below) clustered at the C-terminus of p53 (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998; Liu *et al.*, 1999). It is not known why there are so many lysine residues targeted for acetylation. The precise function of acetylation and the mechanism by which acetylation controls p53 also remain to be established. Acetylation was previously correlated with p53-dependent senescence and its ability to induce apoptosis (Luo *et al.*, 2000; Pearson *et al.*, 2000). As acetylation of p53 enhances its DNA-binding activity *in vitro* (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998), it has been widely assumed that acetylation functions by enhancing p53 transcriptional activity. However, there may be additional mechanisms by which acetylation could control p53 function. For example, since the activation of p53 involves its stabilization, we have previously suggested that acetylation might control the stability of p53. Indeed, our observation that inhibition of p53 deacetylation by the deacetylase inhibitor trichostatin A (TSA) is accompanied by an increase in p53 stability is consistent with this hypothesis (Ito *et al.*, 2001). However, how acetylation controls p53 stability is not known.

In this report, we present evidence that acetylation controls p53 stability by potentially interfering with MDM2-mediated ubiquitylation. We found that MDM2 and the deacetylase HDAC1 form a complex that controls p53 acetylation in a cooperative fashion, thus providing a molecular link between the ubiquitylation and the deacetylation machinery. We provide evidence that stable overexpression of a dominant-negative HDAC1 mutant in fibroblasts leads to markedly enhanced p53 acetylation and p53 stability in response to DNA damage, suggesting that one function of acetylation is to promote p53 stability. Consistent with this conclusion, we found that acetylation and ubiquitylation occur at an overlapping set of lysine residues in p53. Our results suggest a simple model wherein acetylation promotes p53 stability by competing with MDM2-mediated ubiquitylation. The MDM2–HDAC1 interaction thus provides an efficient coupling of deacetylation and ubiquitylation machinery to negatively regulate p53 function.

Results

HDAC1 specifically interacts with MDM2

Our previous studies have shown that MDM2 can inhibit p300-induced p53 acetylation, in part, by repressing p300 acetyltransferase activity. However, the inhibitory effect of MDM2 on p53 acetylation can also be reversed by a pan-HDAC deacetylase inhibitor TSA (Ito *et al.*, 2001), suggesting the involvement of active deacetylation. To further address the potential mechanism by which MDM2 negatively regulates p53 acetylation, we first considered the possibility that MDM2 may induce p53 deacetylation by recruiting a putative p53 deacetylase, since MDM2 itself does not possess TSA-sensitive deacetylase activity (data not shown). To test this hypothesis, we investigated whether MDM2 interacts with any of the known HDAC family members. Through a co-immunoprecipitation assay, we found that among HDAC1–7, only HDAC1 strongly co-immunoprecipitated with MDM2, while the others did not (Figure 1A; data not shown). This result indicates that MDM2 selectively interacts with HDAC1. To determine whether MDM2 binds HDAC1 directly, the ability of bacterially-expressed recombinant GST HDAC1 and MDM2 proteins to interact was investigated by pull-down assay (Figure 1B). As expected, recombinant MDM2 bound GST–p53 (Figure 1B, lane 3); however, MDM2 failed to bind GST–HDAC1 under the same conditions (Figure 1B, lane 2). Thus, these results indicate that MDM2 specifically interacts with HDAC1 via an

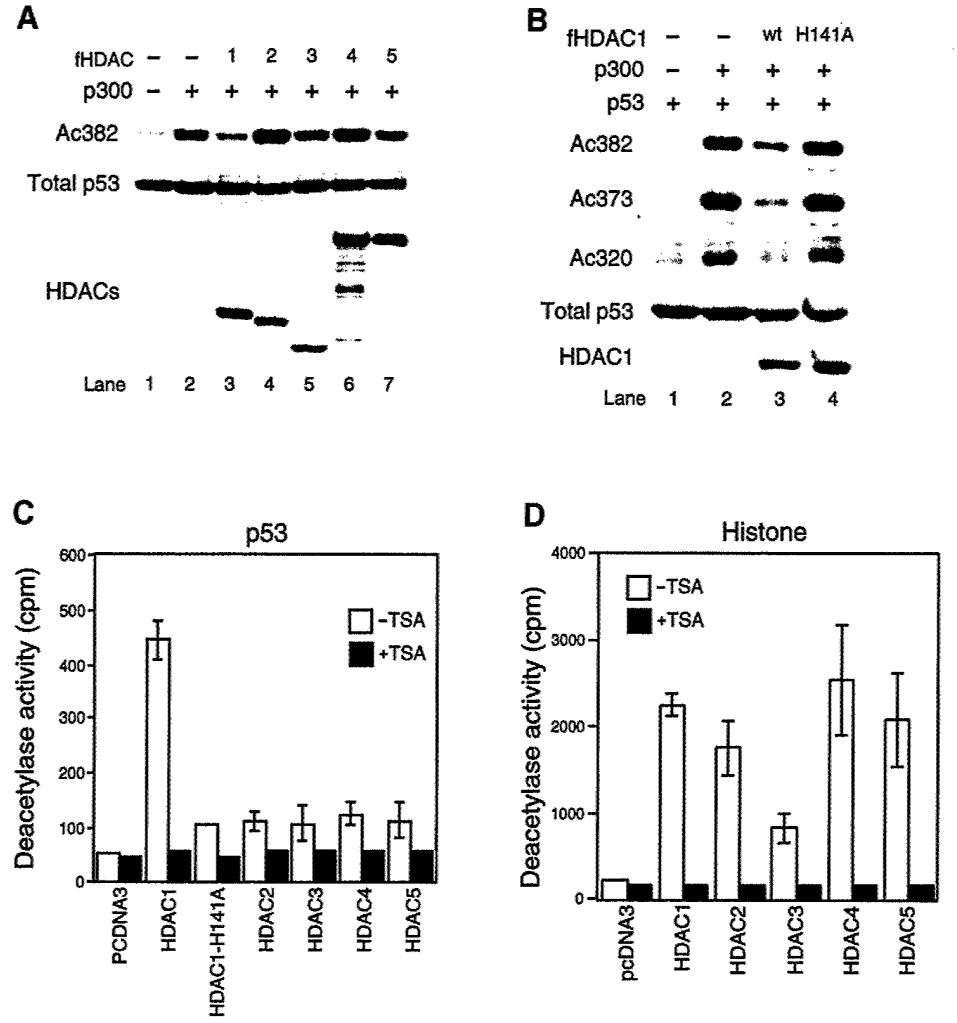

Fig. 2. Deacetylation of p53 by HDAC1. (**A**) 293T cells were transfected with an empty vector (lane 1) or cotransfected with either 2 μg of p300 alone (lane 2) or cotransfected with 2 μg of Flag-tagged HDAC1 (lane 3), 2 μg of HDAC2 (lane 4), 2 μg of HDAC3 (lane 5), 4 μg of HDAC4 (lane 6) or 4 μg of HDAC5 (lane 7). The levels of endogenous acetylated p53, total p53, and each HDAC were detected by immunoblotting with anti-acetylated p53 (Lys382) (top panel), anti-p53 antibody (middle panel) and anti-Flag antibody (bottom panel). (**B**) H1299 cells (p53–/–) were transfected with 0.2 μg of p53 alone (lane 1) or cotransfected with either 2 μg of p300 alone (lane 2), with 2 μg of HDAC1 wild-type (lane 3) or 2 μg of enzyme-dead H141A mutant (lane 4). The level of acetylated p53 was assessed using either antibody specific for acetylated Lys382 (Ac382), acetylated Lys373 (Ac373) or acetylated Lys320 (Ac320). The levels of total p53 and Flag-tagged HDAC1 were detected as described in (A). (**C** and **D**) 293T cells were transfected with empty vector or Flag-tagged HDACs and cellular extracts were prepared as described in Materials and methods. Deacetylase activity was measured against acetylated GST–p53 (C) or histone H4 peptide (D) in the presence or absence of TSA. Results are representative of three independent experiments. Note that all HDAC family members possess deacetylase activity towards histones, but only HDAC1 can efficiently deacetylate p53.

indirect mechanism or that a specific modification of MDM2 or/and HDAC1 is required for this interaction.

As MDM2 binds p53 directly, we investigated whether the binding of MDM2 and HDAC1 was mediated by p53. To test this possibility, an HDAC1 expression plasmid was transfected into H1299 cells (p53–/–) and the interaction of MDM2 and HDAC1 was determined by pull-down assay using GST–MDM2, followed by immunoblotting to visualize the associated HDAC1. As shown in Figure 1C, GST–MDM2, but not GST, interacts with HDAC1 expressed in H1299 cells. A similar conclusion regarding HDAC1–MDM2 interaction in H1299 cells was obtained by co-transfecting HDAC1 and MDM2 expression plasmids, which were assayed by co-immunoprecipitation (Figure 1D). These results demonstrate a specific and p53-independent interaction between MDM2 and HDAC1.

We investigate further the interaction between the endogenous HDAC1 and MDM2. As shown in Figure 1E, at the basal state in which the MDM2 level is low, a co-immunoprecipitation assay revealed a weak but reproducible interaction of MDM2 and HDAC1. However, upon UV irradiation, which induces MDM2 expression (~4-fold), a marked increase of MDM2 and HDAC1 association (~3-fold) was observed. These results demonstrate an endogenous MDM2–HDAC1 interaction that can be stimulated by UV irradiation, likely due to an increase of the MDM2 protein level in response to DNA damage.

MDM2–HDAC1 deacetylates p53 cooperatively

The physical interaction of MDM2 and HDAC1 supports the hypothesis that MDM2 recruits HDAC1 to deacetylate p53. Consistent with this hypothesis, it was previously

proposed that HDAC1 could function as a p53 deacetylase (Luo *et al.*, 2000). However, although the ectopic expression of HDAC1-associated MTA2/PID can induce p53 deacetylation, whether or not HDAC1 is a p53 deacetylase *in vivo* was not directly tested, nor did the study address whether other HDAC family members could deacetylate p53. To investigate these issues and determine whether the specific association with MDM2 is functionally correlated with the ability of an HDAC to function as a p53 deacetylase, expression plasmids for p300, HDAC1–5 were cotransfected into 293T cells and the p53 acetylation status on lysine 382 was assessed using an acetyl-Lys382-specific antibody. As shown in Figure 2A, consistent with its unique association with MDM2, only HDAC1 but not other HDACs efficiently induced p53 deacetylation on lysine 382 (Figure 2A, lanes 3–7). Importantly, ectopic expression of HDAC1 also led to deacetylation at lysine 320 and 373 (Figure 2B), both of which are also known to become acetylated upon p53 activation (Sakaguchi *et al.*, 1998; Liu *et al.*, 1999). Thus, *in vivo,* HDAC1 can deacetylate all three known acetylated lysine residues in p53. An identical conclusion that HDAC1, but not other HDAC members, possesses strong p53 deacetylase activity can be demonstrated directly by an *in vitro* assay using HDACs immunoprecipitated from cells transfected with the HDAC expression plasmids (Figure 2C), while all HDAC family members possess a deacetylase activity toward histones (Figure 2 D). Thus, among the HDAC members tested, HDAC1 uniquely associates with MDM2 and can specifically function as a p53 deacetylase, supporting its role for mediating MDM2-dependent p53 deacetylation.

To determine whether MDM2 is required for HDAC1 to function as a p53 deacetylase, we investigated the ability of HDAC1 to deacetylate p53 in MDM2-deficient cells. As shown in Figure 3A, overexpression of HDAC1 still results in p53 deacetylation in MDM2–/–;p53–/– mouse embryonic fibroblasts (MEFs). Although MDM2 is not required for high levels of HDAC1 to deacetylate p53, it is possible that the interaction with MDM2 could facilitate HDAC1's activity as a p53 deacetylase. To test this possibility, low concentrations of MDM2 and HDAC1 were co-expressed with p53 and p300 in MDM2–/–; p53–/– MEFs, and the p53 acetylation status was assessed. Under these conditions, neither MDM2 nor HDAC1 alone had an appreciable effect on the level of p53 acetylation (Figure 3B, lanes 2 and 4). However, co-expression of both MDM2 and HDAC1 dramatically reduced the level of p53 acetylation (Figure 3B, lane 5). These results demonstrate that MDM2 and HDAC1 function synergistically to induce p53 deacetylation. To elucidate the potential mechanism underlying this synergistic activity, we asked whether MDM2 affects the interaction between p53 and HDAC1. As shown in Figure 3C, in MDM2-deficient MEFs, HDAC1 only interacts weakly with p53 (Figure 3C,

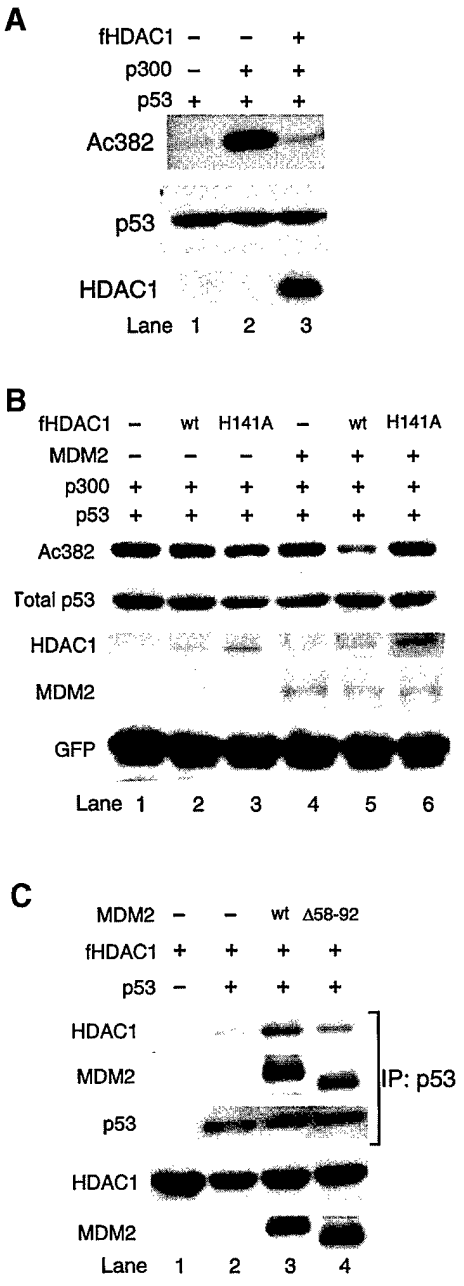

Fig. 3. HDAC1 and MDM2 work cooperatively to deacetylate p53. (**A**) MEF (p53–/–;MDM2–/–) cells were transfected with 0.1 μg of p53 (lane 1), or cotransfected either with 2 μg of p300 alone (lane 2) or with p300 and 2 μg of Flag-tagged HDAC1 (lane 3). The level of total p53 (middle panel) and acetylated p53 (top panel) were detected as described in Figure 2. (**B**) MEF (p53–/–;MDM2–/–) cells were transfected with either 0.1 μg of p53, 2 μg of p300 and 0.5 μg of internal control GFP (lane 1), or cotransfected either with 12.5 ng of Flag-tagged HDAC1 wild-type (lane 2), with 12.5 ng of Flag-tagged HDAC1 H141A mutant (lane 3), with 0.5 μg of MDM2 (lane 4), with MDM2 and 12.5 ng of Flag-tagged HDAC1 wild-type (lane 5) or 0.5 μg of MDM2 and 12.5 ng of Flag-tagged HDAC1 H141A mutant (lane 6). The levels of indicated proteins were determined by immunoblotting. Of note, we used 160× the amount of Flag-tagged HDAC1 expression vectors in (A) when compared with (B). (**C**) MEF (p53–/–;MDM2–/–) cells were transfected with either 1 μg of Flag-tagged HDAC1 wild-type alone, or cotransfected either with 0.3 μg of p53, with 0.3 μg of p53 and 4μg of MDM2 wild-type, or 0.3 μg of p53 and 4μg of p53-binding-deficient MDM2 mutant (Δ58–92). Cells were also treated 24 h post-transfection with the protease inhibitor LLnV (10 μM) for 4 h to inhibit MDM2-mediated p53 degradation. Cellular extracts were immunoprecipitated with anti-goat p53 antibody followed by immunoblotting with Flag antibody (top panel), anti-MDM2 antibody (second panel) or anti-p53 antibody (third panel). Total HDAC1 and MDM2 protein were detected with either anti-Flag antibody (fourth panel) or anti-MDM2 antibody (bottom panel). Of note, the interaction between p53 and p53-binding-deficient MDM2 mutant (Δ58–92) is likely mediated by HDAC1 through the ternary complex formation (second panel, lane 4).

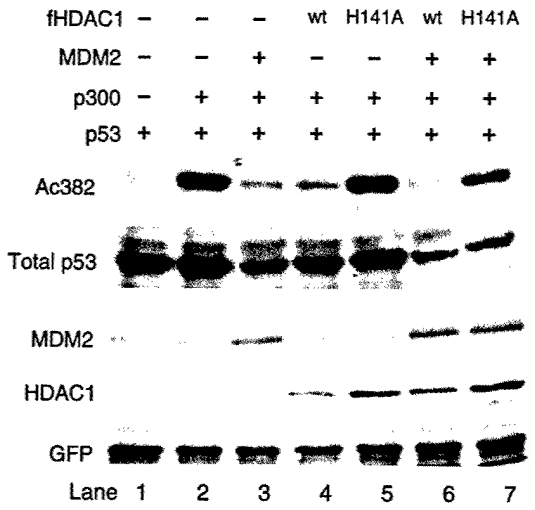

Fig. 4. MDM2 induces p53 deacetylation through HDAC1. H1299 cells were transfected with 0.2 μg of p53 wild-type and 0.5 μg of GFP (all lanes), and cotransfected with one or more of the following; 2 μg of p300 (lanes 2–7), 2 μg of MDM2 (lanes 3, 6 and 7), 2 μg of Flag-tagged HDAC1 wild-type (lanes 4 and 6), 2 μg of Flag-tagged HDAC1 H141A mutant (lanes 5 and 7). The levels of indicated proteins were determined by immunoblotting.

lane 2). However, this interaction is dramatically stimulated upon the re-introduction of wild-type MDM2 (Figure 3C, lane 3). The p53-binding-deficient MDM2 mutant is less efficient in facilitating the p53–HDAC1 interaction. (Figure 3C, lane 4). Thus, the reduced p53 binding is correlated with reduced recruitment of HDAC1 by MDM2. Together, these results indicate that MDM2 promotes the interaction between HDAC1 and p53 and forms a ternary complex, allowing efficient p53 deacetylation.

HDAC1 mediates MDM2-dependent p53 deacetylation

We next examined whether or not HDAC1 was responsible for MDM2-mediated p53 deacetylation. The observation that HDAC1 binds MDM2 indirectly (Figure 1) and that bacterially-expressed recombinant HDAC1 does not possess p53 deacetylase activity (data not shown) suggests that the p53 deacetylase activity of HDAC1 requires additional factors. We reasoned that as HDAC1 normally functions in a multi-protein complex (Zhang *et al.*, 1998a), a catalytically inactive (enzyme-dead) HDAC1 could potentially titrate away the cofactors needed for the active complex and thus function as a dominant-negative mutant. To this end, we asked whether a catalytically inactive HDAC1 mutant (H141A) could reverse the effect of MDM2 on p53 acetylation. As shown in Figure 4, overexpression of MDM2 causes a dramatic reduction of p53 acetylation induced by p300 (Figure 4, lane 3). However, upon co-expression of the HDAC1 H141A mutant, the effect of MDM2 is partially lost and p53 acetylation restored (Figure 4, lane 7). This observation supports the idea that HDAC1 H141A acts as a dominant-negative mutant and that MDM2-induced p53 deacetylation is mediated by HDAC1. Together, these observations provide evidence that MDM2 recruits HDAC1 into a multi-protein complex, which promotes p53 deacetylation.

A common set of lysines are modified by acetylation and ubiquitylation

To begin to address the potential function of p53 acetylation, we first generated p53 mutants that can not be acetylated (Figure 5A). By analyzing the acetylation pattern of recombinant p53 acetylated by CBP, we found that mutation of all three known acetylated lysines (320, 373 and 382) does not eliminate p53 acetylation completely (Figure 5B, lane 2). However, additional mutations of lysines 370, 372 and 381 essentially abolished the acetylation by CBP (Figure 5B, lane 3). Thus, there are at least six lysine residues in p53 that can be acetylated by CBP *in vitro*. To generate the non-acetylatable p53 mutant, all six lysine residues were mutated to arginine (6KR), in order to minimize the structural impact by maintaining the positive charges at these residues.

Our previous study established a positive correlation between p53 acetylation and its total protein level (Ito *et al.*, 2001); we therefore examined the potential function of acetylation in regulating p53 stability. As both acetylation and ubiquitylation modify the ε amino group of lysine residues, we first evaluated whether there is a relationship between p53 acetylation and ubiquitylation. Recent studies on p53 ubiquitylation have identified several lysine residues important for MDM2-mediated ubiquitylation (Nakamura *et al.*, 2000; Rodriguez *et al.*, 2000). When compared with the acetylated lysine residues identified in this report, we found that lysine residues targeted for ubiquitylation overlap those that can be acetylated *in vitro* (Figure 5C). Consistent with this idea, the acetylation-deficient 6KR mutant is completely resistant to MDM2-mediated degradation (Figure 5D and E). Thus, a common set of lysine residues is targeted by both p300/CBP-mediated acetylation and MDM2-mediated ubiquitylation. This result suggests the possibility that the acetylation of C-terminal lysine residues might prevent their ubiquitylation and consequently lead to p53 stabilization.

A dominant-negative HDAC1 mutant promotes both p53 acetylation and stability in response to DNA damage

Despite its ability to affect p53 acetylation levels, HDAC1 and its H141A mutant showed little effect on p53 protein levels when acetylation was induced by overexpression of p300 (Figure 4). It is possible that there may be only a very small portion of total p53 becoming acetylated under this artificial condition, and consequently, the balance of acetylation and ubiquitylation is not faithfully reflected in this system. To circumvent this problem and to investigate the role of p53 acetylation in a physiological setting, we generated NIH 3T3 lines that stably expressed HDAC1 or its dominant-negative mutant by retrovirus-mediated gene transfer. A physiological level of p53 acetylation can be induced by DNA-damaging agents in these cell lines, and p53 stability and activity can then be evaluated. To this end, control NIH 3T3 cells and their derivatives overexpressing wild-type HDAC1 and dominant-negative HDAC1 were irradiated with UV, and the levels of p53 and its acetylation were determined. Importantly, overexpression of HDAC1 or its dominant-negative mutant had no effect on the basal level of p53 or its acetylation (Figure 6A). This indicates that there is little

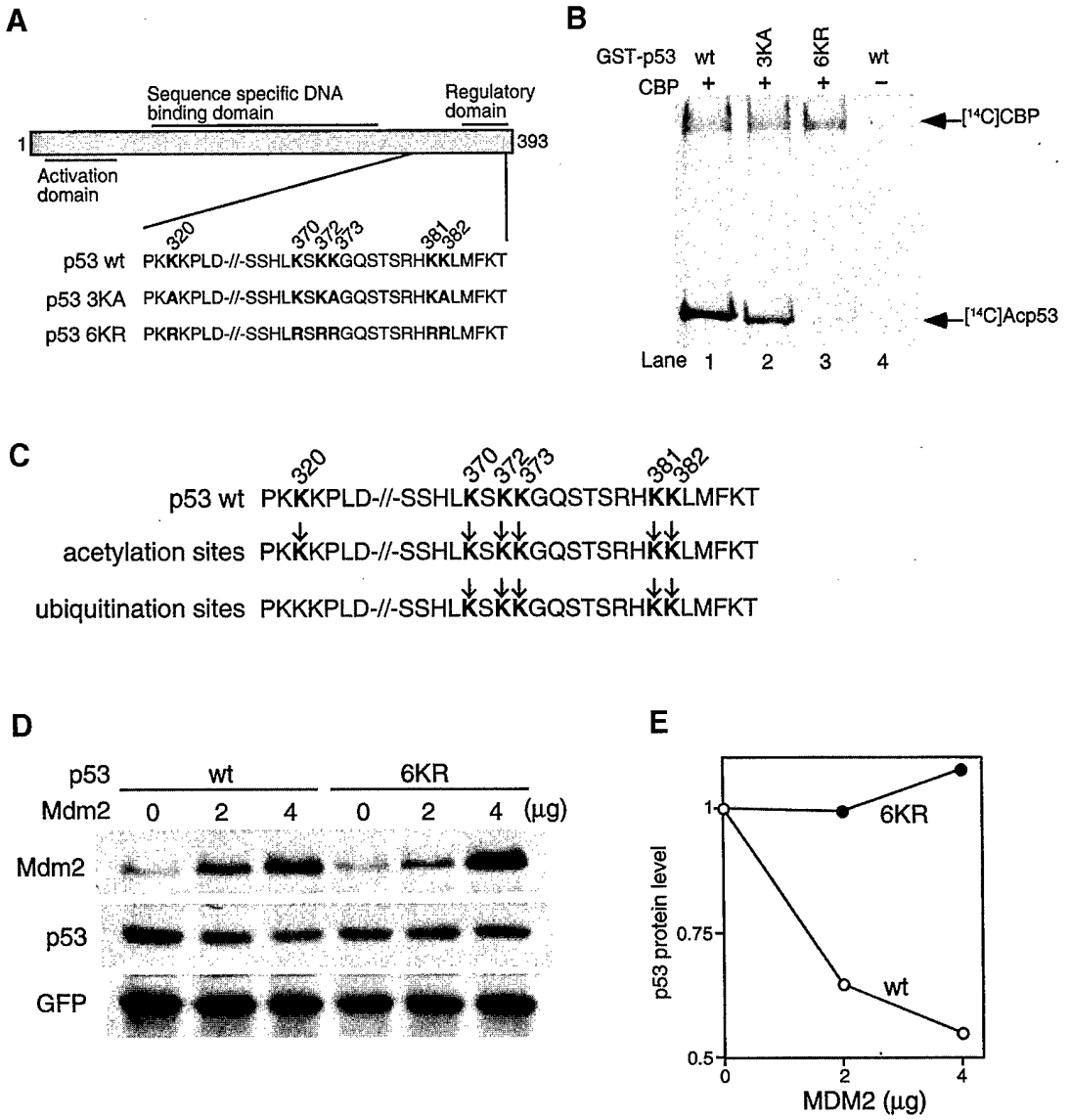

Fig. 5. Mutations of lysine residues of acetylation sites prevent MDM2-mediated p53 degradation. (**A**) Schematic structure of p53 mutations of C-terminal lysines to alanines or arginines. (**B**) GST–p53 wild-type (lane 1), GST–p53 3KA mutant with mutated lysine residues 320, 373 and 382 to alanine residues (lane 2), or GST–p53 6KR mutant with mutated lysine residues 320, 370, 372, 373, 381 and 382 to arginine residues (lane 3) were acetylated by recombinant CBP in the presence of the [^{14}C]acetyl-CoA and analyzed by SDS–PAGE followed by autoradiograph. Acetylated p53 and CBP are indicated by arrows. As a negative control, wild-type GST–p53 was incubated without recombinant CBP in the presence of the [^{14}C]acetyl-CoA (lane 4). (**C**) Acetylation sites of p53 overlap with ubiquitylation sites. The lysine residues susceptible to acetylation and ubiquitylation in the C-terminus of p53 are indicated by arrows. (**D**) H1299 cells were transfected with 0.5 μg of an expression plasmid encoding GFP as an internal control, 0.2 μg of wild-type p53 or p53 6KR mutant, together with empty pcDNA3 vector or the indicated amount of MDM2 vector. Thirty-six hours after transfection, cell extracts were prepared and analyzed by immunoblotting with anti-p53, anti-MDM2 and anti-GFP antibodies. (**E**) The band intensity of p53 and GFP protein levels was measured with NIH imaging software. p53 (empty circles) and 6KR mutant (filled circles) levels were normalized to GFP levels and were set to 1 in the absence of MDM2.

non-specific effect on p53 activation caused by HDAC1 or its mutant. After UV treatment, however, the p53 acetylation level was significantly enhanced in NIH 3T3 cells overexpressing dominant negative HDAC1 and reduced in the wild-type HDAC1-expressing cells (Figure 6A and C). This result demonstrates that HDAC1 can modulate p53 acetylation status in response to DNA damage. Consistent with the hypothesis that acetylation promotes p53 stability, the level of stabilized p53 protein is dramatically increased in the H141A mutant cell line in response to UV irradiation (Figure 6A and B). This conclusion is further supported by the observation of an increase in p53 half-life in mutant HDAC1 H141A-expressing cells and a decrease in wild-type HDAC1-expressing cell when compared with control cell lines (Figure 6D and E). Importantly, the UV-induced, p53-dependent induction of p21 and MDM2 also reaches a much higher level in HDAC1 H141A mutant cell lines than in control and wild-type HDAC1 lines, demonstrating that an increase in p53 acetylation is accompanied by both

p53 stabilization and enhanced function (Figure 6A and B). Together, these results support the idea that HDAC1 regulates p53 stability and function by modulating its acetylation levels.

Discussion

We have demonstrated previously that acetylation of p53 is invariably and transiently induced upon its activation (Ito *et al.*, 2001). The transient nature of p53 acetylation suggests the presence of negative regulators for p53 acetylation. In this report, we identify MDM2 and HDAC1 as the key components that function cooperatively to control p53 deacetylation. We found that acetylation functions, at least in part, by promoting p53 stability. The realization that p300/CBP-mediated acetylation and MDM2-mediated ubiquitylation occur at a common set of lysine residues provides a potential molecular mechanism by which acetylation controls p53 stability by competing with ubiquitylation. The identification of an MDM2–HDAC1 interaction thus provides a novel mechanism to couple the regulation of acetylation and ubiquitylation for the efficient control of p53 levels.

We have previously demonstrated that p53 acetylation is negatively regulated by MDM2 and that this activity can be reversed by $p14^{ARF}$ (Ito *et al.*, 2001). We and others have shown that MDM2 suppresses p53 acetylation, at least in part, by inhibiting the acetyltransferase activity of p300 and CBP (Kobet *et al.*, 2000; Ito *et al.*, 2001). However, we also observed that the inhibitory activity of MDM2 toward p53 acetylation can be reversed by the deacetylase inhibitor TSA (Ito *et al.*, 2001). This observation led us to propose that MDM2 must utilize additional mechanisms to regulate p53 acetylation. The identification of a specific interaction between MDM2 and HDAC1 now provides evidence for a second mechanism employed by MDM2 to control p53 acetylation. Consistent with the idea that MDM2 recruits HDAC1 to downregulate p53 acetylation, HDAC1, but not other members of the HDAC family (2, 3, 4, 5 and 7), has the capacity to function as a p53 deacetylase towards all three known acetylated lysine residues of p53 *in vivo* (Figure 2; data not shown). Interestingly, HDAC2 has been found to co-exist with HDAC1 in several complexes (Zhang *et al.*, 1998a), but does not interact with MDM2 appreciably and has no significant p53 deacetylase activity. This surprising observation raises an interesting possibility that HDAC1 might reside in a different complex to function as a p53 deacetylase. An earlier report demonstrated that HDAC1 acts as a p53 deacetylase based on the observation that overexpression of HDAC1-associated PID/MTA-2 protein can induce p53 deacetylation (Luo *et al.*, 2000). Our study, however, provides the first direct evidence that HDAC1 indeed functions as a p53 deacetylase *in vivo* (Figure 2). It is worth noting that our data suggest that MDM2 and HDAC1 may interact via an intermediate factor; however, given the important role of PID/MTA-2 in p53 deacetylation (Luo *et al.*, 2000), it remains to be tested whether PID plays a role in mediating the HDAC1–MDM2 interaction. The important role of HDAC1 in MDM2-mediated p53 deacetylation is further substantiated by the finding that a dominant-negative HDAC1 mutant (H141A) can restore p53 acetylation levels in the presence of MDM2 (Figure 4). The identification of an MDM2–HDAC1 complex not only provides a novel mechanism by which p53 acetylation is regulated, but it also revealed an unexpected link between the acetylation and ubiquitylation machinery (see below for more discussion).

Although high levels of HDAC1 can induce p53 deacetylation in the absence of MDM2, low levels of HDAC1, which likely reflect physiological conditions, fail to do so (Figure 3A and B). However, the ability of HDAC1 to function as a p53 deacetylase, at low levels, is dramatically induced in the presence of MDM2 (Figure 3B). This result suggests that MDM2 facilitates the functional interaction between HDAC1 and p53. In support of this hypothesis, we showed that the physical association between HDAC1 and p53 was enhanced in the presence of MDM2 (Figure 3C). As the level of HDAC1 itself appears to be constant and not subject to regulation (Figure 1E; A.Ito, unpublished observation), the interaction with MDM2, whose level is regulated by p53 in response to various stresses, provides a means to control the activity of HDAC1 toward p53. Under this scenario, the p53 deacetylase activity of HDAC1 becomes activated when the MDM2 levels are increased by active p53 in response to stress. Consistent with this idea, the endogenous interaction between HDAC1 and MDM2 is enhanced after DNA damage when the level of MDM2 is induced (Figure 1E). Thus, together with MDM2, HDAC1 becomes a key component of a p53 negative feedback loop. Supporting this idea, inhibition of HDAC1 activity by an HDAC1 dominant-negative mutant leads to a dramatic enhancement of DNA damage-induced p53 acetylation, p53 stability and activity (Figure 6). The cooperative activity of MDM2 and HDAC1 toward p53 could be achieved through the stimulation of p53–HDAC1 complex formation as shown in Figure 3C. Another interesting possibility is that, as MDM2 can ubiquitylate substrates other than p53 (Shenoy *et al.*, 2001), MDM2 might regulate the activity of HDAC1 by ubiquitylating HDAC1 or a component of the HDAC1 complex. Such a possibility is consistent with our previous observation that $p14^{ARF}$, which is known to inhibit the MDM2 E3 ligase activity toward p53 (Honda and Yasuda, 1999), can reverse MDM2-mediated p53 deacetylation (Ito *et al.*, 2001).

Regardless of the mechanism, our results support the idea that the recruitment of HDAC1 by MDM2 plays an important role in regulating p53 deacetylation and function. However, it is likely that HDAC1 is not the only deacetylase that regulates p53 acetylation. We have evidence that a portion of p53 deacetylation could be carried out in the cytoplasm whereas HDAC1 resides in the nucleus (A.Ito, Y.Kawaguchi and T.P.Yao, unpublished observation). Furthermore, recent studies have shown that the NAD-dependent and TSA-insensitive histone deacetylase Sir-2, can also deacetylate p53 (Luo *et al.*, 2001; Vaziri *et al.*, 2001). However, unlike HDAC1, which is able to deacetylate p53 at all three known acetylated Lys residues, Sir-2 was shown to mainly deacetylate Lys 382 (Vaziri *et al.*, 2001). Further studies will be required to elucidate the individual role of these deacetylases in regulating p53 function. However, the participation of multiple deacetylases further supports the

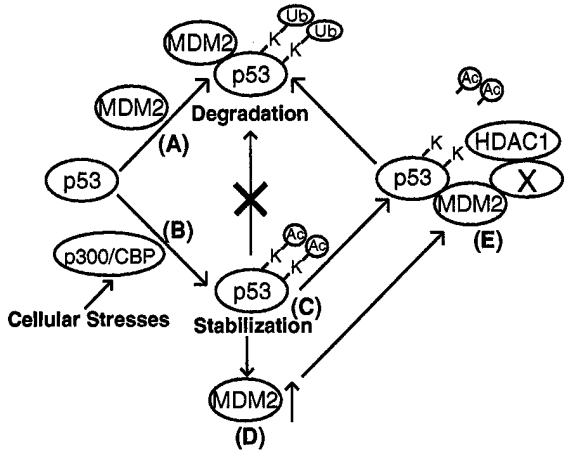

Fig. 7. A model for the regulation and interplay of p53 acetylation and ubiquitylation. Under unstressed conditions, p53 is ubiquitylated on lysine residues by MDM2 and targeted for degradation (A). Upon its activation by various cellular insults, p53 becomes acetylated by p300/CBP at the same set of lysine residues also targeted by MDM2 (B). Acetylation on lysine residues thus prevents MDM2-mediated ubiquitylation and leads to p53 stabilization (C). The stabilized p53 functions as a tumor suppressor and also induces MDM2 (D). The high level of MDM2, in turn, recruits the p53 deacetylase HDAC1 and triggers p53 deacetylation (E). The deacetylated p53 with unmodified lysine residues is now ready to be ubiquitylated by MDM2 and ultimately degraded.

in the recruitment of transcriptional co-activators (Barlev *et al.*, 2001). Our current study provides evidence that, in addition to a role in p53 transcriptional activity, there exists another novel function for p53 acetylation. We have reported previously the parallel kinetics of p53 acetylation and stabilization and the enhancement of p53 stability by inhibition of its deacetylation (Ito *et al.*, 2001). Both observations are consistent with the idea that one function of acetylation is to promote p53 stability. By assessing the p53 status in fibroblasts stably overexpressing a dominant-negative mutant of HDAC1, we have obtained further evidence that enhanced acetylation is associated with an increase in p53 protein stability in response to DNA damage (Figure 6). Although the complete *in vivo* acetylation sites in p53 still remain to be established, the *in vitro* mapping effort reveals that both p300/CBP-mediated acetylation (Figure 5B) and MDM2-mediated ubiquitylation (Nakamura *et al.*, 2000; Rodriguez *et al.*, 2000) occurred at an overlapping set of lysine residues (Figure 5C). This observation offers a further link between p53 acetylation and stability. The potential competition for acetylation and ubiquitylation of these lysine residues provides a plausible molecular mechanism by which acetylation promotes p53 stability. In this model (Figure 7), at the p53 basal state, a set of C-terminal lysine residues of p53 are subject to MDM2-mediated ubiquitylation, which leads to p53 degradation. Upon its activation by stress signals, these lysine residues become acetylated by p300/CBP and are no longer available for MDM2-dependent ubiquitylation, leading to p53 stabilization. Stabilized p53 functions as a tumor suppressor and induces high levels of MDM2, which in turn, promotes p53 deacetylation by recruiting a p53 deacetylase, HDAC1. The unmodified lysine residues can then serve as the substrates for MDM2-mediated ubiquitylation resulting in p53 degradation. The interaction of MDM2 and HDAC1 provides a novel molecular mechanism for an efficient coupling of deacetylation and ubiquitylation of p53 that allows MDM2 to effectively inactivate and degrade p53 and complete the negative feedback loop. The potential functional interaction between the acetylation and ubiquitylation machinery described in this model would also suggest a broader and more general role for reversible acetylation in the regulation of protein stability and other ubiquitylation-dependent biological processes.

Materials and methods

Cell lines and transfection

293T, H1299, A549 and MDM2–/–;p53–/– double null MEF cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, while NIH 3T3 cells were maintained in DMEM supplemented with 10% calf serum. All cells were grown at 37°C in a humidified atmosphere of 5% CO_2. All transfections were performed by either the calcium phosphate method as described previously (Yao et al., 1992) or the lipofectamine method (Invitrogen). Retroviruses were produced by transient transfection of a pBabe Puro vector construct into phoenix cells. NIH 3T3 cells were infected with retrovirus containing media in the presence of 8 μg/ml of polybrene overnight. Thirty-six hours later, cells were selected in the presence of 1.5 μg/ml puromycin and kept under selection in medium containing 1.5 μg/ml puromycin during experiments.

Plasmids

p53, MDM2, and p300 vectors were described previously (Ito *et al.*, 2001). HDAC3 cDNA was provided by Dr F.Dangond (Harvard Medical School) and cloned into the *Eco*RI/ *Not*I sites of pcDNA3 vector with a Flag tag. HDAC4 and 5 cDNAs were provided by Dr S.L.Schreiber (Harvard University) and described in Grozinger *et al.* (1999). HDAC1 H141A mutant cDNA (provided by Dr S.L.Schreiber) was subcloned into the *Bam*HI/*Eco*RI sites of pcDNA3 vector. For retroviral constructs, HDAC1 wild-type and H141A mutant cDNAs were cloned into the *Bam* HI/*Eco*RI sites of the pBabe Puro vector. p53–3KA mutant cDNA, mutated lysines 320, 373 and 382 to alanine, 6KR mutant, and mutated lysines 320, 370, 372, 373, 381 and 382 to arginine cDNAs were generated by site-directed mutagenesis and cloned into the pcDNA3 or the pGEX vector (Amersham).

DNA-damage treatment

DNA damage was performed by exposing cells to a 310 nm wavelength UV source and cells were harvested at indicated time points.

Immunoprecipitation and immunoblotting

Cells were lysed in buffer (20 mM Tris–HCl pH 7.6, 170 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM DTT) supplemented with 5 μM TSA and protease inhibitors. For immunoprecipitation with anti-goat p53 antibody, equal amounts of lysate (containing 100–300 μg of total cellular protein) were incubated with 1 μg of goat anti-p53 antibody (FL-393; Santa Cruz) and protein G–Sepharose (Pharmacia) for 3 h at 4°C. To detect acetylated mouse p53, equal amounts of lysate (containing 300–500 μg of total cellular protein) were incubated with agarose-conjugated anti-p53 antibody (Pab421) overnight at 4°C. To detect endogenous MDM2 and HDAC1 interaction, 2 mg of cellular lysate was incubated with 1 μg of anti-MDM2 antibody (SMP14; Santa Cruz) and protein A and G–Sepharose mixtures (Pharmacia) overnight at 4°C. When immunoprecipitation was not performed, 20–50 μg of total extracts were analyzed. Proteins were detected by chemiluminescent ECL kit (Amersham) with one of the following antibodies: anti-human p53 antibody (Ab-6; Calbiochem), anti-p53 antibody for detecting mouse p53, anti-human acetylated (Lys320) p53 antibody (Sakaguchi *et al.*, 1998), anti-human acetylated (Lys373) p53 antibody, anti-human acetylated (Lys382) p53 antibody (Calbiochem), anti-mouse acetylated (Lys382) p53 antibody, anti-human MDM2 antibody (SMP14; Santa Cruz), anti-Flag antibody (M2; Sigma), anti-HDAC1 antibody (H-11; Santa Cruz), anti-α-tubulin antibody (DM1A; Sigma), anti-p21 antibody (H164; Santa Cruz) or anti-GFP antibody (Boehringer Mannheim).

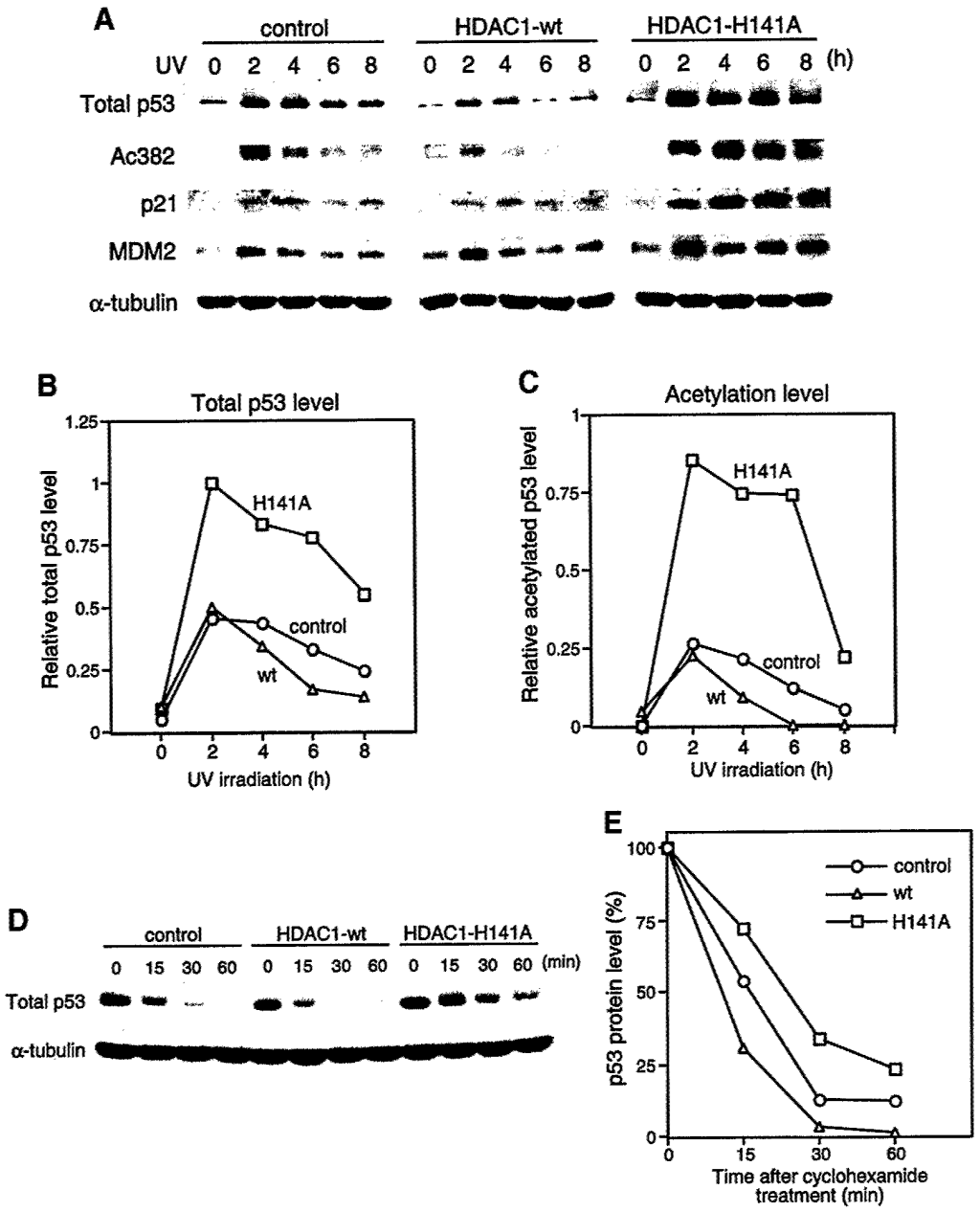

Fig. 6. Effect of HDAC1 on p53 acetylation, stability and activity in response to DNA damage. (**A**) NIH 3T3 cells infected with mock vector (control), pBabe-HDAC1 wild-type (HDAC1-wt), or pBabe-HDAC1 H141A mutant (HDAC1 H141A) were exposed to UV-B (75 J/m^2). Cells were harvested at the indicated times. The levels of total p53 (panel 1), acetylated p53 (panel 2), p21 (panel 3), MDM2 (panel 4) and the internal control α-tubulin (panel 5) were assessed by immunoblotting. (**B** and **C**) The band intensity of p53, acetylated p53 and α-tubulin protein levels in all three cell lines were measured with NIH imaging software. The levels of p53 (B) and acetylated p53 (C) were normalized to α-tubulin and the highest intensity levels of p53 or acetylated p53 were set to 1. (B) and (C) are representative results of three (B) and two (C) independent experiments. (**D**) All three stable cell lines were exposed to UV-B (75 J/m^2) and 2 h post-irradiation, cyclohexamide (10 μg/ml) was added to inhibit new p53 protein synthesis (designated 0 h). Cells were harvested at the time points indicated after cyclohexamide treatment. The level of total p53 (upper panel) and α-tubulin (lower panel) was determined. (**E**) The band intensity of p53 and α-tubulin protein levels were measured by NIH imaging software. p53 levels were normalized to α-tubulin levels and calculated against the amount of p53 present at time point 0, which was set at 100%. Results are representative of three independent experiments.

idea that acetylation is a critical mechanism for regulation of p53.

We have previously shown that acetylation appears to be a critical modification, as it invariably accompanies p53 activation and is the target of key p53 regulators, like MDM2, p14^{ARF} and p300/CBP (Ito *et al.*, 2001). However, the exact function and mechanism by which acetylation controls p53 activation remains unclear. As acetylation stimulates p53 DNA-binding activity *in vitro* (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998), it was hypothesized that acetylation of p53 promoted its transcriptional activity. Supporting this idea, a recent study shows that, although it does not play a major role on p53 association with target promoters *in vivo*, p53 acetylation is involved

In vitro deacetylation assay

The expression vectors for Flag-tagged HDACs (10–15 μg) were transfected into 293T cells and the cells were lysed in low stringency buffer (50 mM Tris–HCl pH 7.5, 120 mM NaCl, 0.5 mM EDTA, 0.5% NP-40) in the presence of protease inhibitors. After pre-clearing with protein A beads, the extracts were immunoprecipitated with anti-Flag antibody in the presence of rabbit anti-mouse antibody and protein A beads for 5 h at 4°C and then the beads were washed three times with low stringency buffer, twice with low stringency buffer containing 0.5 M NaCl, and twice with deacetylase buffer (10 mM Tris–HCl pH 8.0, 10 mM NaCl, 10% glycerol). For inhibition studies, the immune complexes were pre-incubated with 400 nM of TSA in deacetylase buffer for 30 min at 4°C. The immune complexes were incubated with 10 000 c.p.m. of ^{14}C-labeled acetylated GST–p53 or ^{3}H-labeled acetylated histone 4 peptide in 200 μl of deacetylase buffer for 2 h at 37°C and the release of [^{14}C] or [^{3}H]acetate was quantified by scintillation counting.

In vitro acetylation assay

The *in vitro* acetylation assay was performed as described previously (Ito *et al.*, 2001). Briefly, recombinant CBP protein (1 μg), purified from sf9 insect cells infected with baculovirus expressing CBP, was incubated with 1 μg of wild-type GST–p53 or GST–p53 mutants in the presence of 50 nCi [^{14}C]acetyl-coenzyme A in 30 μl of reaction buffer (50 mM Tris–HCl pH 8.0, 10% glycerol, 1 mM dithiothreitol, 100 μM EDTA, 1 mM PMSF) for 1 h at 37°C. Acetylation was analyzed by SDS–PAGE followed by autoradiography.

Acknowledgements

We thank Dr S.L.Schreiber for his generous gift of the HDAC1 H141A, HDAC4 and HDAC5 cDNAs, Dr F.Dangond for the HDAC3 cDNA, Dr C.W.Wu for the GST–HDAC1 construct and Dr G.Lozano for the MDM2–/–;p53–/– double null MEF cells. We are grateful to Drs C.Anderson, B.Harvat, A.R.Means, X.F.Wang, Ms A.Guardiola and Mr T.A.Bolger for critically reading the manuscript. This work is supported by funding from the Damon Runyon-Walter Winchell Cancer Foundation (DRS 20), National Institutes of Health (CA85676-01A1) and Department of Defense (DAMD 17-01-1-0054) to T.-P.Y.

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Received January 17, 2002; revised and accepted September 30, 2002

Classification: Biological Science/Cell Biology

Charge modification of C-terminal lysine residues controls p53 subcellular localization

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Manuscript information:

Word and character counts:

Abbreviations footnotes:

Abstract

At its basal state, the tumor suppressor p53 is negatively regulated by MDM2- mediated ubiquitination at specific lysines, which leads to p53 nuclear exclusion and degradation. Upon activation, however, these lysines become acetylated by p300/CBP resulting in p53 stabilization. Here we report a surprising finding that p300-mediated acetylation also promotes cytoplasmic localization of p53. Over-expression of p300, but not an acetyltransferase-deficient mutant, stimulates the cytoplasmic accumulation of p53, an effect negated by inactivating its C-terminal nuclear export signal (NES), and by the nuclear export inhibitor LMB. p300-dependent cytoplasmic accumulation of p53 does not involve MDM2, but requires the lysines targeted by p300. Mechanistically, conversion of a minimal four lysines to alanines but not arginines mimics the effect of MDM2 and p300 on the cytoplasmic accumulation of p53 by preventing p53 tetramerization, and thus exposes its NES. Our results suggest that ubiquitination and acetylation regulate p53 transport by neutralizing the lysine-dependent charge patch, which in turn controls p53 oligomerization and the accessibility of the NES. Thus, the charge patch created by C-terminal lysines is the critical determinant and target for the regulation of p53 subcellular localization.

Introduction

The tumor suppressor p53 plays critical roles in regulating cell growth and protecting cells from malignant transformation. Due to this potent growth inhibitory and pro-apoptotic activity, p53 is kept inactive at low concentrations in unstressed cells and any activation of p53 must be transient. In general, the negative regulation of p53 requires the oncogene MDM2 (1). MDM2 can bind p53 directly and suppress p53 transcriptional activity ((2-4)). MDM2 also regulates p53 protein stability by serving as an E3 ligase for p53 ubiquitination, which triggers the rapid degradation of p53 (5-8). The induction of MDM2 by p53 is believed to form the basis of a negative feedback loop that ensures p53 activation is transient (9). However, a recent study showed that, in response to specific types of stresses, the activation of p53 does not lead to MDM2 induction (10). This observation suggests the existence of an alternative mechanism that is capable of terminating p53 activity.

The activity of p53 is also regulated by its subcellular localization. p53 actively shuttles between the nucleus and cytoplasm via a mechanism that is mediated by a nuclear export signal (NES) and its receptor Crm-1 (reviewed in (11). Although its importance for p53 function is apparent, little is known about how p53 nuclear export is regulated. Recently, p53 export was reported to be activated by MDM2-mediated ubiquitination (12-15). However, the exact mechanism by which ubiquitination promotes p53 nuclear export is not known. It has been also proposed that the C-terminal NES is normally exposed in the inactive monomeric or dimeric forms of p53, which are subject to active nuclear export. Upon activation, however, p53 forms tetramers, in which the C-terminal NES is buried and inaccessible to the Crm1 export machinery, resulting in p53

nuclear retention (16). At present, it is not understood how the oligomerization status of p53 is regulated in response to specific stress signals or modifications, such as ubiquitination, that control p53 function.

In contrast to ubiquitination, acetylation has been generally believed to play a positive role in p53 function (reviewed in (17)). We have shown that various p53-activating agents promote p53 acetylation, which is correlated with increased stability (18). The acetylation of p53, which is catalyzed by the p300/CBP acetyltransferases, occurs on at least six lysine residues clustered at the C-terminal (18-21) (A.I. and T.P.Y, submitted). It is unclear why so many lysines are modified by acetylation. Nor is it known whether the acetylation of the lysines provides a functional moiety for protein interaction or serves to modify the conformation of p53. One clue as to the function of p53 acetylation lies in several potentially interesting links between acetylation and ubiquitination. For example, both the acetylation and ubiquitination machinery modify the ε amino group of the lysine residue. Furthermore, acetylation and ubiquitination occur on a common set of lysine residues at the C-terminus of p53 (A.I. and T.P.Y. submitted). Lastly, MDM2 negatively regulates p53 acetylation (18, 22). From these observations, we hypothesize that MDM2-mediated ubiquitination and p300/CBP-mediated acetylation target the same set of lysine residues in p53 to control its function.

Here, we report the surprising observation that, in contrast to its usually positive role in p53 function, p300-mediated acetylation also promotes p53 nuclear exit leading to p53 accumulation in cytoplasm. We show that over-expression of p300 stimulates cytoplasmic accumulation of p53 in an acetylation-dependent but MDM2-independent manner. Mechanistically, we provide evidence that both acetylation and ubiquitination

promote redistribution of p53 to cytoplasm by neutralizing the "charge patch" created by the C-terminal lysine residues. We further show that charge neutralization of the C-terminal lysines prevents p53 oligomerization, which would lead to the exposure of the p53 NES, and thus allowing efficient p53 nuclear export. The requirement of multiple modified lysines for efficient export suggests a potential novel threshold mechanism wherein the acetylation level of p53 serves as a signal that terminates p53 function by activating its nuclear export.

Results

Acetylation regulates subcellular localization of p53

We have previously shown that p53 becomes acetylated by p300/CBP upon activation (18). As p53 activation is accompanied by its accumulation in the nucleus, we asked whether p300-mediated acetylation regulates p53 subcellular localization. To test this, we transfected p53 null H1299 cells with expression plasmids for p53 and p300, and then examined the subcellular localization of p53. As shown in Figure 1A(a), when expressed alone, p53 resides almost exclusively in the nucleus. To our surprise, upon co-expression with p300, in the majority of cells that expressed both transfected p53 and p300, p53 localized to both the nucleus and cytoplasm (Figure 1A(b), and B). Cell counts demonstrate that up to 84% of cells that express both p53 and p300 showed cytoplasmic accumulation of p53 (Figure 1B). To determine if the accumulation of p53 in the cytoplasm involves p53 acetylation, we first examined whether an acetyltransferase-deficient p300DYmutant (C.H. Lai, T.P.Y. in preparation) can also modify the subcellular localization of p53. As shown in Figure 1A(c) and B, unlike wild type p300, the acetyltransferase deficient p300DYmutant did not stimulate p53 accumulation in the

cytoplasm. Importantly, neither p300 nor p300DY mutant has apparent effect on the levels of p53 under the experimental condition (Figure 1D, and (18). Together, these results indicate that p300-mediated cytoplasmic accumulation of p53 requires acetyltransferase activity and is therefore likely mediated by specific acetylation events, such as p53 acetylation.

As p53 subcellular localization is regulated by active nuclear export, we investigated whether acetylation-dependent p53 accumulation in the cytoplasm is concerned with activation of nuclear export. As shown in Figure 1A(d) and B, the nuclear export inhibitor leptomycin B (LMB) efficiently blocked the cytoplasmic accumulation of p53 induced by p300. We also evaluated the subcellular localization of a mutant p53 whose C-terminal nuclear export signal (NES) is inactivated (16). As shown in Figure 1A(f) and B, p53 nuclear export mutant no longer responds to p300 and remains in the nucleus. These results demonstrate that p300-mediated cytoplasmic accumulation of p53 requires C-terminal NES and suggest that acetylation might enhance p53 nuclear export.

To further verify this conclusion from the immuno-localization study, we determined the subcellular localization of p53 in response to p300 by biochemical fractionation. As shown in Figure 1C, p53 is normally a nuclear protein (Lane 1-2). However, co-expression of wild type p300, but not the p300DY mutant, induced a marked accumulation of p53 protein in the cytoplasmic fraction (compare lane 2, 4 and 6). The p300-dependent cytoplasmic accumulation of p53 can again be largely reversed by LMB treatment (Lane 8). These results are in agreement with the immuno-localization study, providing further evidence that p300-mediated acetylation affects subcellular localization of p53. Importantly, acetylated p53 is present in the cytoplasmic fraction

(Figure 1C). Thus, p53 can be exported to the cytoplasm after becoming acetylated, which is consistent with the idea that p300-mediated p53 acetylation promote p53 nuclear export.

p300-mediated cytoplasmic accumulation of p53 is independent of MDM2

It was reported that MDM2 stimulated p53 nuclear export by promoting p53 ubiquitination (12, 13). As p300 functionally interacts with MDM2 (23), we determined whether p300-induced cytoplasmic accumulation of p53 requires MDM2. To test this idea, we expressed p53 alone or together with p300 in MDM2-deficient MEF cells (24), and assessed the subcellular localization of p53. As shown in Figure 2A, in the absence of MDM2, p300 is still capable of stimulating the cytoplasmic accumulation of p53. In fact, more than 90% of p300-over-expressing cells showed p53 in cytoplasm, which is similar to the effect induced by MDM2 (Figure 2B). This result indicates that p300-mediated cytoplasmic accumulation of p53 is independent of MDM2~~, and suggests that p300 can efficiently induce p53 nuclear export with MDM2 independent machinery~~.

C-terminal lysine residues are required for efficient acetylation-induced cytoplasmic accumulation of p53

The results presented so far support the idea that p300 affects subcellular localization of p53 in an acetylation-dependent manner, likely by directly acetylating p53. To further investigate this possibility, we determined whether lysine (K) residues known to be acetylated by p300 are required for acetylation-induced cytoplasmic accumulation of p53. We mutated multiple lysines to arginines (5KR and 6KR, Figure 3A) that are known targets of acetylation by p300/CBP. We then evaluated the subcellular distribution of these mutants in response to p300. As shown in Figure 3B,

when expressed alone, the localization of these p53 KR mutants is almost entirely nuclear and indistinguishable from wild type p53. However, in response to ectopically expressed p300, the number of cells that show a cytoplasmic accumulation of the 5KR and 6KR p53 mutants is markedly reduced when compared with that of wild type p53. These observations indicate that the lysine residues acetylated by p300 are required to mediate a maximal p53 nuclear exit in response to p300.

Neutralization of positively charged lysine residues in the C-terminus regulates subcellular localization of p53

Our data suggests that, similar to ubiquitination, the acetylation of p53 also leads to its accumulation in the cytoplasm. As already discussed, acetylation and ubiquitination modify an over-lapping set of lysine residues (Figure 3 and A.I. and T.P.Y. submitted). However, ubiquitin and acetyl groups have little in common structurally other than the fact that both modify the ε amino group of the lysine residue, consequently neutralizing its charge. We hypothesize that acetylation and ubiquitination promote p53 nuclear exit by neutralizing the charge of the targeted lysine residues. To test this hypothesis, we generated charge-neutralizing mutations by converting lysine (K) residues known to be acetylated and ubiquitinated to alanine (A), either individually or in combination, and evaluated their subcellular localization. As shown in Figure 4, the subcellular localization of the 2KA and several 3KA (3KA-1, 3KA-2 and 3KA-3) mutants with different combination of lysine residues mutated (Figure 4A), are similar to that of wild type p53 and are largely nuclear (Figure 4B and C). In contrast, when four lysine residues are mutated in three different combinations (4KA-1, 4KA-2 and 4KA-3), these p53 mutants clearly began to accumulate in the cytoplasm. The conversion of five lysine residues

(5KA) leads to a further increase in the number of cells with cytoplasmic p53 staining (Figure 4B and C). The cytoplasmic localization of the 4KA-1, 4KA-2, 4KA-3 and 5KA p53 mutants were observed in 51%, 52%, 48% and 64% of transfected cells, respectively, compared with 19% for wild type p53 (Figure 4C). Furthermore, the intensity of the wild type p53 detected in the cytoplasm is much weaker than those of 4KA and 5KA mutants (data not shown). In agreement with the immuno-localization results, quantitation by subcellular fractionation showed that 31% of the total 4KA mutant p53 is localized to the cytoplasm while the level of cytoplasmically localized wild type p53 is almost undetectable by this assay (Figure 4D). Importantly, the cytoplasmic populations of the p53 4KA and 5KA mutants can be effectively eliminated upon treatment with LMB, supporting the idea that the 4KA and 5KA mutations lead to the activation of p53 nuclear export. Together, these observations indicate that the degree of p53 cytoplasmic accumulation is proportional to the number of lysine residues neutralized. Therefore, these results suggest that ubiquitination and acetylation modulate p53 subcellular localization by modifying the charge patch created by the lysine residues at the C-terminus of p53.

C-terminal lysine charge patch determines the oligomerization status of p53

The accessibility of the C-terminal NES to the export machinery has been proposed to be regulated by the oligomerization status of p53 (16). We therefore assessed whether modification of the lysine charge activates p53 export by regulating p53 oligomerization status. The observation that the conversion of four but not three lysines to alanines activates p53 export provides a convenient system to address this question. To test this idea, recombinant wild type, 3KA, and 4KA (4KA 1-3, Figure 4A) mutant

polypeptides encompassing the entire p53 tetramerization domain and lysine rich C-terminus (amino acids 326-393) were tested for their ability to ologmerize. A mutant p53 with four lysines converted to arginines instead of alanine (4KR), which prevents ubiquitination and acetylation but preserves the charge of the lysine, was used as an additional control. As shown in Figure 5, the wild type and 3KA polypeptides dimerized and tetramerized readily (Lanes 2 and 4). In contrast, the 4KA-1, 4KA-2 and 4KA-3 polypeptides completely failed to do so (Lane 6, 8 and 10). This observation is in perfect agreement with their respective sub-cellular localizations (Figure 4). Importantly, polypeptides from the 4KR mutant, which is a nuclear protein (data not shown), show a wild type capacity to oligomerize (Lane 12). This result further supports the hypothesis that charge modification is the key element in the regulation of p53 oligomerization. Together, these results demonstrate that the oligomerization status of p53 can be controlled by the charge conferred by a defined number of lysine residues.

Discussion

In this report, we provide evidence that p53 acetylation regulates p53 subcellular localization, at least in part, by activating nuclear export. This surprising finding suggests that acetylation might have an unsuspected role in the termination of p53 signaling. Our attempt to understand how two unrelated modifications, namely acetylation and ubiquitination, both stimulate p53 nuclear exit led to the discovery that the charge patch presented by the C-terminal lysine residues plays a critical role in regulating p53 subcellular localization. Modification of lysine residues by either ubiquitination or acetylation neutralizes this charge patch, which in turns prevents p53 oligomerization and exposes the nuclear export signal, leading to efficient export. Our study provides a

unified model for the understanding how p53 nuclear export is regulated by ubiquitination and acetylation.

The regulation of p53 subcellular localization is believed to be controlled, in part, by its oligomerization status. The regulation of p53 nuclear export is likely centered on the accessibility of the NES. Interestingly, the dominant C-terminal NES is located in the oligomerization domain of p53 and it was previously suggested that the accessibility of the p53 NES is dependent on its oligomerization status (16). This hypothesis fits with the observation that the active form of p53 assumes a teterameric conformation that will mask the NES and allow efficient nuclear accumulation for p53 to function in the nucleus(25) (26) (27) (28). This conclusion is also supported by the solution and the crystal structure of the oligomerization domain, which demonstrates that the C-terminal NES is exposed in monomeric or dimeric conformations but it is buried in the p53 tetramers (29-31). The inter-conversion between the tetrameric and monomeric or dimeric states would therefore determine the availability of the NES and, consequently, the efficiency of p53 export. However, the biochemical basis that controls p53 oligomerization and its regulation were not known. In this report, we provide the experimental evidence that the charge provided by lysine residues at the C-terminus is the key determinant of p53 oligomerization status, which could in turn controls the efficiency of p53 nuclear export. This conclusion is supported by the observation that mutations that neutralize specific lysine residues result in p53 mutants that cannot oligomerize and have a more prominent cytoplasmic localization. Importantly, this cytoplasmic accumulation of p53 can be reversed by LMB treatment, further suggesting a dominant role of p53 nuclear export in response to charge modification (Figure 4 and 5). Our study, however,

did not exclude the possibility that charge neutralization of lysine residues might also affect p53 nuclear import as well. Under such a scenario, acetylated or ubiquitinated p53 will be trapped in the cytoplasm due to inefficient nuclear import. Regardless which mechanism might play a more dominant role, our results support the idea that charge modification of the lysine residues dictates the subcellular localization of p53.

If C-terminal charge plays a critical role in p53 subcellular localization, how is this charge patch regulated? Evidence suggests that MDM2-mediated ubiquitination, which was recently shown to promote p53 export (14, 15), is one key regulator. In theory, the ubiquitin moiety itself could be the signal that activates p53 nuclear export, as is the case for its role in receptor endocytosis (reviewed in (32)). However, the conclusion that acetylation, which shares no structural similarity with ubiquitin, can nonetheless promote cytoplasmic accumulation of p53 by activating export machinery indicates that this hypothesis is highly unlikely. Rather, our observation that the mutation of C-terminal lysines to alanines, but not arginines, leads to p53 cytoplasmic accumulation (Figure 4 and 5), supports the hypothesis that ubiquitination as well as acetylation promotes p53 export by neutralizing the charge of the lysine residues. A similar cytoplasmic accumulation of p53 by lysine to alanine mutation was also observed by other studies ((33) (14)). Consistent with this observation, and the hypothesis that the charge presented by lysine is the critical determinant for p53 export activity, the conversion of key lysine residues to isoleucine also leads to the cytoplasmic accumulation of p53 (15).

Interestingly, although the conversion of three different combinations of four lysines known to be targets of acetylation and ubiquitination to alanines (4KA mutant) promotes cytoplasmic accumulation of p53, conversion of three lysines at different

combinations (3KA) has little effect (Figure 4). This result suggests that there might be a threshold for the activation of p53 export, which is determined by the overall charge provided by the lysine cluster at the C-terminus. Supporting this hypothesis, we showed that the p53 4KA mutant fails to tetramerize, while the 3KA mutant oligomerizes as efficiently as the wild type (Figure 5). This result correlates well with the respective subcellular localization of these mutants and further supports the proposed model that the p53 NES is not accessible in the teterameric configuration but exposed in momomeric or dimeric forms (16). The NES in the 4KA p53 mutant is therefore more accessible to Crm1 than that in the wild type or 3KA mutant, resulting in more active export of the p53 4KA but not 3KA mutant. The idea that acetylation regulates p53 export by neutralizing charge concurs with an elegant study on histone H2A.Z in which acetylation was shown to regulate the histone tails by modifying a charge patch of multiple lysines (34). Thus, our analysis suggests that a similar charge-dependent mechanism mediated by lysines is used to control p53 oligomerization and its subcellular localization.

We note however that the p53 4KA and 5KA mutants are more resistant to MDM2-mediated degradation(33), and our data not shown. Thus, although K to A mutations can mimic the effect of ubiquitin-induced p53 nuclear export, they do not confer the ubiquitin-mediated p53 degradation. This observation has two implications. First, p53 nuclear export and degradation are not necessarily linked events. A similar conclusion is drawn from a recent study showing that a p53 mutant deficient in nuclear export can nonetheless be degraded by MDM2 (15). Second, it suggests that although the p53 species targeted for degradation and nuclear export are both modified by ubiquitin, they likely represent two distinct p53 populations that are modified differently. As it was

recently demonstrated that MDM2 can promote p53 mono-ubiquitination in vitro (35), a very interesting possibility is that mono-ubiquitinated p53 is exported while the poly-ubiquitinated species is targeted to the proteasome for degradation. How MDM2 promotes two different types of ubiquitination on p53 is a critical issue to be addressed in future studies.

The finding that acetylation regulates subcellular localization of p53, at least in part, by activating nuclear export seems, at first, paradoxical, as all evidence suggests that acetylation positively regulates p53 function (reviewed in (17)). However, a recent study of ubiquitination in transcription provided an unexpected conclusion that ubiquitination not only serves to degrade and inactivate transcriptional activators, but also essential for their transcriptional activity (36). In this case, ubiquitination functions as both a positive and negative modification, providing the efficient coupling of the activation and inactivation of a specific transcription event. By analogy, our findings raise an interesting possibility that acetylation not only activates p53 function initially (18), but also serves as a signal to terminate p53 function by activating its export. In this rather speculative scenario, the number of lysine residues acetylated will constitute the threshold for p53 export, such that when more than four lysine residues are acetylated, p53 will be efficiently exported. Indeed, we have observed acetylated p53 actively exported from the nucleus (Figure 1C).

Our results provide the first evidence that the charge status presented by a specific set of lysine residues is the key determinant and regulatory target for the regulation of subcellular localization of p53, likely by activating nuclear export. The regulation of p53 export is then achieved by the modification of these lysine residues by the MDM2-

mediated ubiquitination and/or p300/CBP-mediated acetylation machinery. As MDM2 is not always induced upon p53 activation (10), our finding that acetylation can regulate subcellular localization of p53 might provide another potential negative feedback mechanism to ensure the activation of p53 is transient and properly terminated.

Acknowledgements

We thank Dr. G. Lozano for MDM2-/-;p53-/- null MEF cells. We are grateful to Dr. D. Lew, Ms. C. Hubbert and A. Guardiola for critically reading the manuscript. This work is supported by funding from the Damon Runyon-Walter Winchell Cancer Foundation (DRS 20), National Institute of Health (CA85676-01A1), and Department of Defense (DAMD 17-01-1-0054) to T.P.Y.

Materials and methods

Cell lines and transfection

H1299 human cells and p53(-/-), MDM2(-/-) mouse embryonic fibroblast (MEF) cells were maintained in Dulbecco's modified Eagle's medium (DMEM). All cells were grown at 37 C in the presence of 10% fetal bovine serum and penicillin/streptomycin in a humidified atmosphere of 5% CO2. All transfections were performed by the calcium phosphate method as described previously (37).

Plasmids

Wild-type human p53 cDNA, wild–type human MDM2, wild-type human myc-p300 and human p300DY mutants were described previously (18). p53-5KR and -6KR mutants were generated using site-directed mutagenesis, changing lysine 320, 370, 372, 373, 381 and 382 to arginine. P53-2KA, -3KA, -4KA and –5KA mutants were also constructed using site-directed mutagenesis to exchange lysine to alanine. p53-NES(-) mutants were constructed using site-directed mutagenesis to change leucine (a.a 348 and 350) to alanine.

Immuno-fluorescence

For immuno-fluorescence staining, cells grown on glass coverslip were transfected with 0.1 μg of p53 and 1μg myc-epitope tagged p300 expression plasmids. Immunostaining was performed as described (38) using anti-p53 rabbit polyclonal antibody (FL-393, Santa Cruz), anti-myc monoclonal antibody 9E10 and anti-MDM2 monoclonal antibody SMP-14 (Santa Cruz).

Fractionation, Immunoprecipitation and immunoblotting

The cells were homogenized using dounce homogenizer in buffer [25 mM Hepes-HCl pH 7.4, 250 mM sucrose, 1 mM EDTA, 5mM MgCl2, 50 mM NaF, 1mM dithiothreitol (DTT)] supplemented with 5 μM of deacetylase inhibitor TSA (Sigma) and protease inhibitors. After centrifugation at 960g for 5 min, nuclei pellets were washed with homogenaize buffer, and then lysed in buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM DDT] supplemented with 5 μM TSA and protease inhibitors. Lysates were centrifuged at 5000g for 5 min to obtain supernatants of nuclear fraction. While, cytoplasmic supernatants were added NaCl and NP-40 to bring up to 150 mM and 1%, respectively. Immunoprecipitation and immunoblotting were performed as described previously (18). Proteins were detected with one of the following antibodies: anti-human p53 antibody (Ab-6, Calbiochem), anti-human acetylated (Lys320) p53 antibody, anti-human acetylated (Lys373) p53 antibody, anti-human acetylated (Lys382) p53 antibody (Calbiochem), anti-α-tubulin antibody (DM1A, Sigma) or anti-green fluorescent protein (GFP) antibody (Boehringer Mannheim).

Protein production and oligomerization assay

Wild-type, 3KA, 4KA or 4KR p53 C-terminal DNA (amino acid 326-393) including the tetramerization domain was cloned into pGEX-6P-1 vectors. GST fusion constructs were expressed in E.coli, and then purified by Glutathione Sepharose 4B (Amersham) and cleaved with prescission protease (Amersham). The oligomerization assay was performed as described previously (16).

Figure legend

Figure 1. Acetylation regulates p53 nuclear export. **(A)** Subcellular localization of p53, p300, and the p300DY mutant following transfection into p53 (-/-) H1299 cells. Cells were transfected with p53 wild-type alone (a, g), p53 wild-type and myc-p300 (b, d, h, j), p53 wild-type and myc-p300DY (c, i), p53 NES(-) mutant alone (e, k), or p53 NES(-) mutant and myc-p300 (f, l) as indicated. The localization of p53, p300, and p300DY was determined by immunostaining with the anti-p53 polyclonal antibody or anti-myc monoclonal antibody (9E10). 10 ng/ml of LMB was added 8h prior to immunostaining as indicated (d, j). **(B)** Acetylation by p300 increases the percent of cells with cytoplasmic p53. 100-200 cells from each transfection were scored. Results are an average of three independent experiments. In the case of co-expression with p300 or p300DY, only anti-myc antibody positive cells were scored. **(C)** Cytoplasmic p53 is acetylated. Levels of acetylated p53 in nuclear (N) and cytolpasmic(C) fraction were determined by immunopreciptation with anti-p53 polyclonal antibody, followed by immunoblotting with anti-acetylated p53 antibody. Levels of total p53 and α-tubulin were assay by blotting with anti-p53 monoclonal antibody (Ab-6) and anti-tubulin monoclonal antibody. **(D)** p300 dose not change expression level of total p53. GFP was used as internal control, and detected by anti-GFP monoclonal antibody.

Figure 2. Acetylation-mediated p53 nuclear export is not MDM2-dependent. **(A)** Subcellular localization of p53, p300 and MDM2 following transfection into p53(-/-) / MDM2(-/-) MEF cells. p53 (a,b,c) and p300 (d) were detected as described in Figure 1A. MDM2 (e) was detected by immunostaining with the anti-MDM2 monoclonal antibody

(SMP-14). **(B)** The percent of cells with cytoplasmic p53 was determined as described in Figure 1B.

Figure 3. The C-terminal lysines of p53 are involved in the acetylation-mediated nuclear export of p53. **(A)** Schematic representation of wild-type p53 and an expanded view of the C-terminus indicating the lysine (K) to arginine (R) mutations of the p53, 5KR, and 6KR mutants. Abbreviations are as follows: TAD, transactivation domain (1-43 amino acids); DNA-BD, DNA binding domain (102-292 amino acids); OLIGO, origomerization domain (326-358 amino acids) **(B)** The loss of available acetylation sites results in a reduction in the percentage of cells with cytoplasmic p53. Wild-type p53 or the KR mutants were transfected into H1299 cells alone or co-transfected with p300 as indicated. The percent of cells with cytoplasmic p53 was determined as in Figure 1B.

Figure 4. Neutralization of positively charged lysine residues in the C-terminus of p53 promotes p53 nuclear export. **(A)** Schematic representation of the p53 lysine (K) to alanine (A) mutants. **(B)** Subcellular localization of wild-type p53 and KA mutants transfected into H1299 cells. LMB treatment was performed as described in Figure 1A. **(C)** An increase in the number of KA mutations results in an increase in the percent of cells with cytoplasmic p53. Results were determined as in Figure 1B. **(D)** The level of cytoplasmic p53 is proportional to the number of lysine residues neutralized. Levels of p53 in nuclear (N) and cytoplasmic(C) fraction were determined as in Figure 1C. The ratio of cytoplasmic p53 is calculated by dividing cytoplasmic p53 by total p53 (nuclear plus cytoplasmic p53).

Figure 5. Neutralization of positively charged lysine residues in the C-terminus determines the oligomerization status of p53. Wild-type (lane 1, 2), 3KA (lane 3, 4), 4KA

(lane 5, 6) or 4KR (lane 7, 8) mutant p53 polypeptides consisting of the tetramerization domain (amino acid 326-393) were expressed in *E.coli* as GST fusions. Ten micrograms of GST-cleaved protein was incubated at 37 C with or without 0.1% glutaraldehyde for 15 min, then analyzed by a 20% SDS-PAGE to distinguish the p53 monomer from oligomers.

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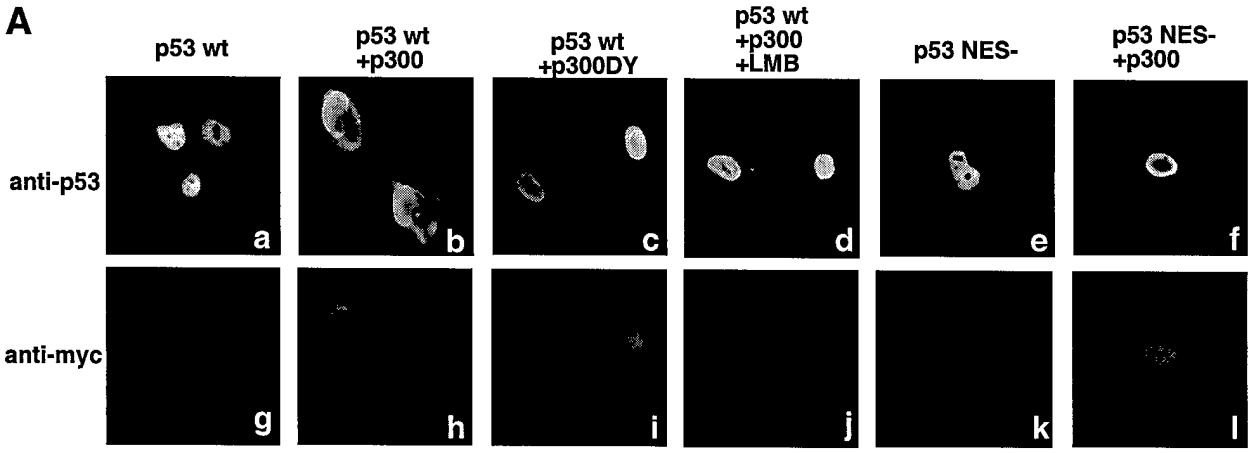
A
p53 wt
p53 wt +p300
p53 wt +p300DY
p53 wt +p300 +LMB
p53 NES-
p53 NES- +p300
anti-p53
anti-myc
a
b
c
d
e
f
g
h
i
j
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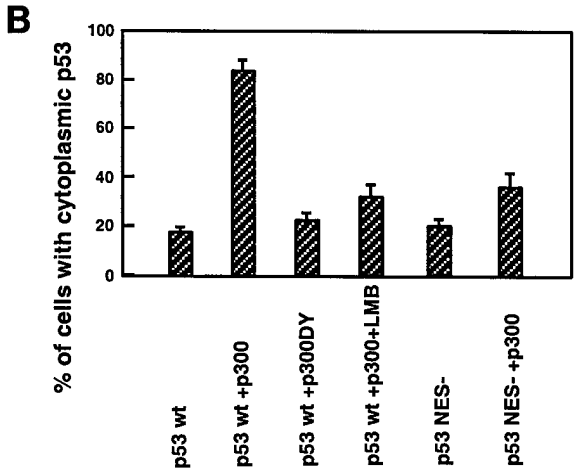
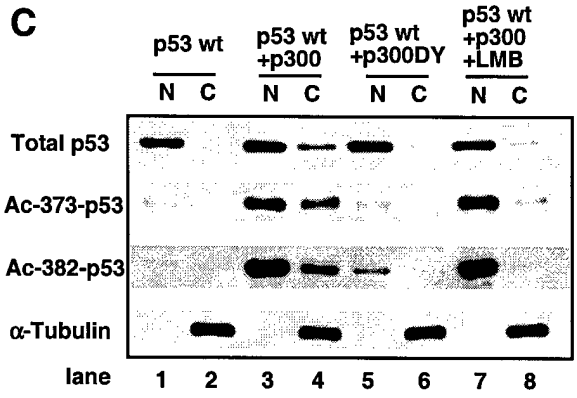
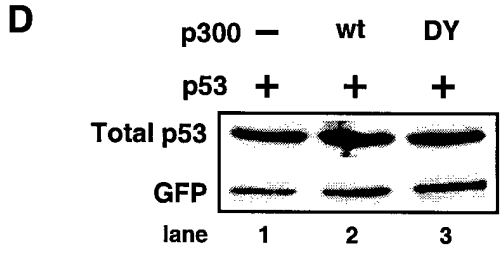
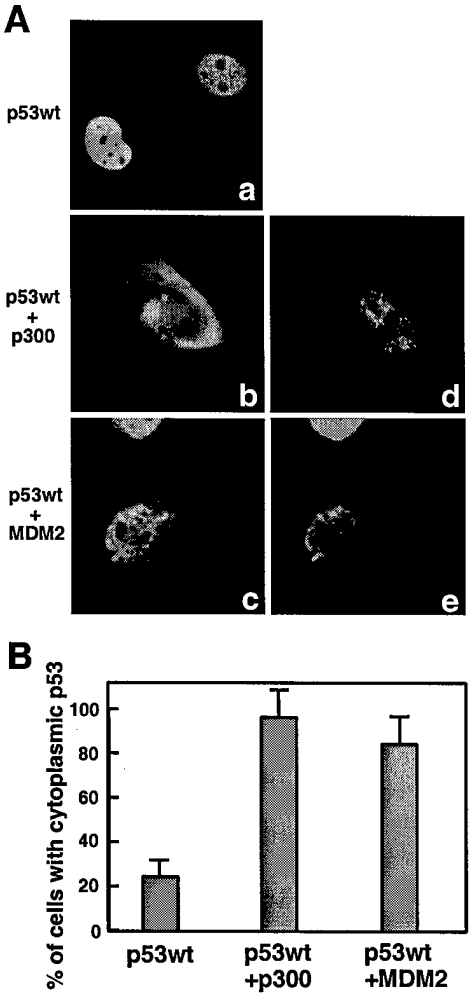
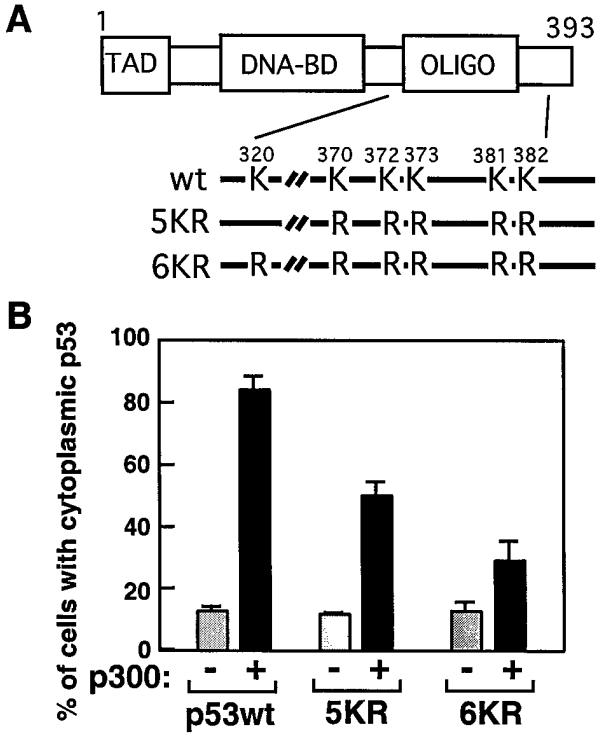
l

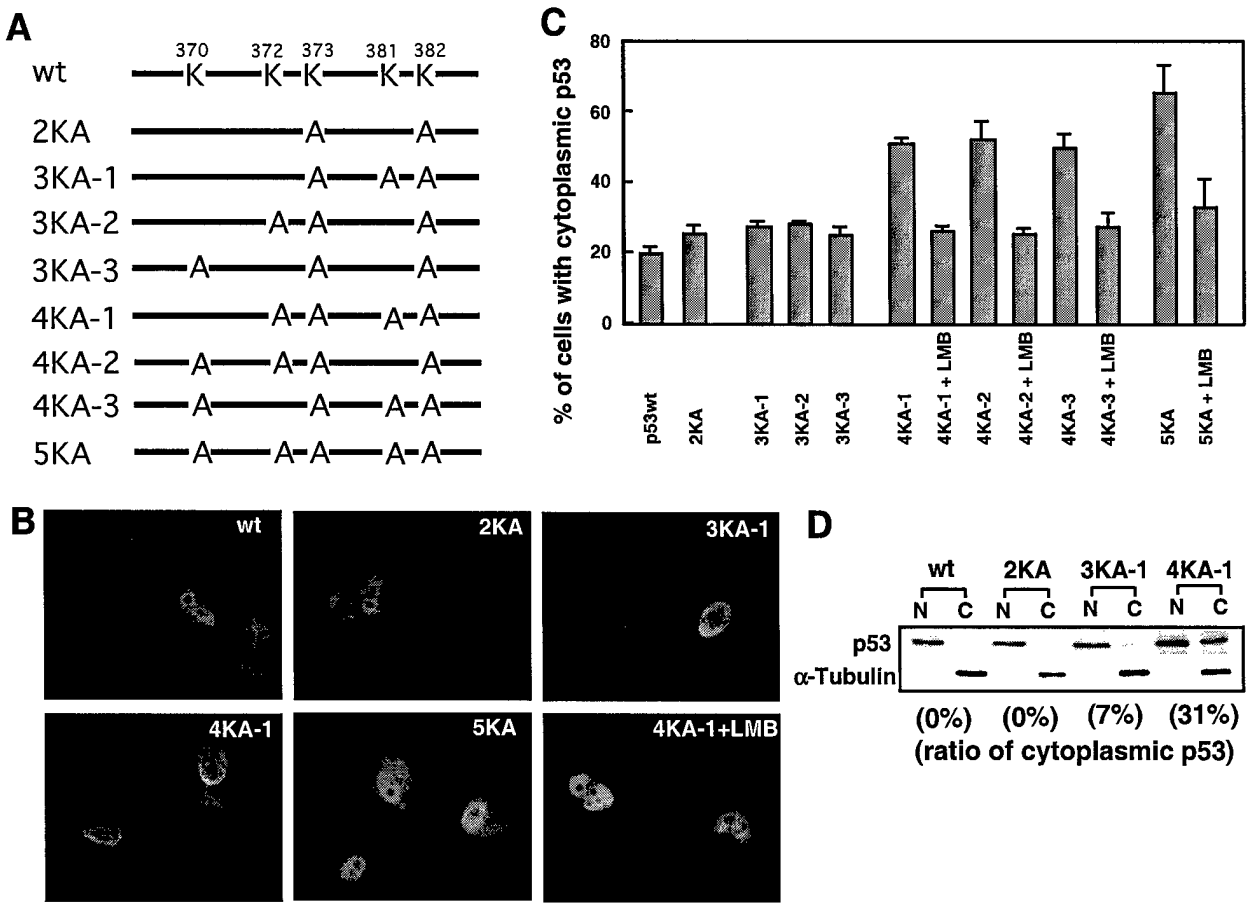

B
% of cells with cytoplasmic p53
100
80
60
40
20
0
p53 wt
p53 wt +p300
p53 wt +p300DY
p53 wt +p300+LMB
p53 NES-
p53 NES- +p300


C
p53 wt
p53 wt +p300
p53 wt +p300DY
p53 wt +p300 +LMB
N C N C N C N C
Total p53
Ac-373-p53
Ac-382-p53
α-Tubulin
lane 1 2 3 4 5 6 7 8


D
p300 – wt DY
p53 + + +
Total p53
GFP
lane 1 2 3


A
p53wt
a
p53wt
+
p300
b
d
p53wt
+
MDM2
c
e
B
% of cells with cytoplasmic p53
100
80
60
40
20
0
p53wt
p53wt
+p300
p53wt
+MDM2


A
1
393
TAD
DNA-BD
OLIGO
320
370
372
373
381
382
wt
5KR
6KR
B
% of cells with cytoplasmic p53
100
80
60
40
20
0
p300:
-
+
p53wt
5KR
6KR


A
370 372 373 381 382
wt K K K K K
2KA A A
3KA-1 A A A
3KA-2 A A A
3KA-3 A A A
4KA-1 A A A A
4KA-2 A A A A
4KA-3 A A A A
5KA A A A A
C
% of cells with cytoplasmic p53
80
60
40
20
0
p53wt
2KA
3KA-1
3KA-2
3KA-3
4KA-1
4KA-1 + LMB
4KA-2
4KA-2 + LMB
4KA-3
4KA-3 + LMB
5KA
5KA + LMB
B
wt
2KA
3KA-1
4KA-1
5KA
4KA-1+LMB
D
wt 2KA 3KA-1 4KA-1
N C N C N C N C
p53
α-Tubulin
(0%) (0%) (7%) (31%)
(ratio of cytoplasmic p53)

Kawaguchi et. al, Figure5